REMARKS

Claims 39-41, 43, 74, 77-79, 85-107 and 109-111 are pending. Claims 1-38, 42, 44-73, 75-76, 80-84 and 108 are canceled without prejudice.

Applicants submit that no new matter has been added as a result of this amendment.

Applicants submit that claims 39-41, 43, 74, 77-79 and 85-107 are rejected for alleged obviousness over WO 97/15310. Applicants also submit that claims 74 and 109-111 are rejected for alleged lack of novelty in view of WO 97/15310. The instant claims are directed to an isolated nestin-positive, GLP-1R-positive or nestin and GLP-1R positive human pancreatic or liver stem cell.

Applicants have identified a post-filing U.S. Patent (U.S. 6,436,704; attached herein as Exhibit A) that includes the following claim: "A substantially pure population of human pancreatic progenitor cells, wherein said population of pancreatic progenitor cells will differentiate into acinar, ductal and islet cells." The phrase "substantially pure population of pancreatic progenitor cells" is defined in the '704 patent at column 7, lines 15 through 19 as "comprised at least about 85% pancreatic progenitor cells, preferably at least about 90% , and even more preferably about 95% or more."

The inventors of the '704 patent submitted an Information Disclosure Statement on August 9, 2000 (attached as Exhibit B) that included the WO 97/15310 PCT application cited by the Examiner in the instant application. Although Examiner Woitach of Art Unit 1632 signed the Information Disclosure Statement on April 10, 2001, the '310 Application was not cited during the prosecution of the '704 patent.

Applicants respectfully submit that it is impossible to conclude that the instant claims to "an isolated nestin-positive, GLP-1R positive or nestin and GLP-1R positive human pancreatic or liver stem cell" are obvious in view of the '310 patent while claim 1 of the '704 patent to "a substantially pure population of human pancreatic progenitor cells, wherein said population of pancreatic progenitor cells will differentiate into acinar, ductal and islet cells" is not. Further, Applicants respectfully submit that it is impossible to conclude that the instant claims to "an isolated nestin-positive, GLP-1R positive or nestin and GLP-1R positive human pancreatic or

liver stem cell" lack novelty over the '310 application while claim 1 of the '704 patent to "a substantially pure population of human pancreatic progenitor cells, wherein said population of pancreatic progenitor cells will differentiate into acinar, ductal and islet cells" does not.

Applicants would appreciate discussing this matter with Examiners Belyavskyi and Woitach.

Rejection of Claims 39-41, 43, 77-79, and 85-107 under 35 U.S.C. §103(a)

Claims 39-41, 43, 74, 77-79, and 85-107 are rejected under 35 U.S.C. §103(a) for alleged obviousness over WO 97/15310.

Applicants respectfully traverse the rejection.

For the reasons described below, Applicants respectfully submit that the Examiner has failed to establish a *prima facie* case of obviousness under the requirements of 35 U.S.C. § 103(a). To establish a prima facie case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings (*In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991)). Second, there must be a reasonable expectation of success. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on Applicants' disclosure. Finally, the prior art reference (or references when combined) must teach or suggest ***all the claim limitations***. *In re Royka*, 490 F.2d 981, 180 U.S.P.Q. 580 (C.C.P.A. 1974).

No reasonable expectation of success in arriving at the claimed invention. Unexpected results.

Applicants submit that even if the '310 application were to present a prima facie case of obviousness with respect to the instant claims (which it does not), one of skill in the art would not have a reasonable expectation of success in arriving at the invention as claimed. That is, the subject matter of the instant claims is **unexpected**.

Evidence of unexpected results in found in the accompanying Rule 1.132 declaration by Dr. Joel Habener in which evidence is presented (Exhibit C) demonstrating that persons of skill in the art (that is reviewers of a JDRF grant application based on work performed in Dr. Habener's laboratory characterizing pancreatic stem cells (review dated May 27, 2003) **did not believe** in the existence of a population of isolated nestin-positive pancreatic stem cells. That is Exhibit C demonstrates **significant doubt by one of skill in the art** as to the existence of nestin-positive pancreatic stem cells.

The reviewers make the following statements that demonstrate clearly the reviewers' disbelief regarding a population of isolated nestin-positive pancreatic stem cells:

- "The reviewers felt that overall the submission of the application is largely premature due to a fundamental lack of supporting preliminary data that is required to support the **novel and somewhat controversial hypothesis presented**."
- "This proposal is based on works performed in the laboratory of Dr. Habener. While the work published by Dr. Habener is interesting, it is **not yet fully accepted by the scientific community**. For example, works performed by the group of Helena Edlund in Sweden or Luc Bouwens in Belgium strongly suggest that nestin-positive cells present in the pancreas are not pancreatic stem cells."
- "If human pancreatic stems cells could be used as a starting source to generate human beta cells, **this would be a dream**."
- "The major problem with this study is that there are at least two publications last year...which very thoroughly have investigated this working hypothesis. Both of them concluded from their data, quite in contrast to the studies by Habener et al. that they call for caution in trying to generate beta cells from nestin-positive cells."
- "The proposal addresses an important issue but for the reasons given above one has to be quite **skeptical** as regards the scientific basis for the study."

Additional evidence of unexpected results discussed in the accompanying Rule 1.132 declaration by Dr. Habener is presented (Exhibit D) demonstrating that persons of skill in the art (that is reviewers of Dr. Habener's manuscript relating to the claimed subject matter of the

instant application) did not believe that a population of isolated nestin-positive stem cells existed. That is, Exhibit D demonstrates significant doubt by one of skill in the art as to the existence of an isolated population of nestin-positive stem cells.

The Examiner asserts that the Peck et al. reference does not specifically teach an isolated nestin-positive or GLP-1R positive human pancreatic or liver stem cell that is not a neural cell. The Examiner asserts that the cells described in the Peck application are "obviously" nestin-positive or GLP-1R positive since the cell population taught in Peck is identical to that claimed in the instant application.

Applicants submit herewith a Rule 1.132 declaration by Dr. Gordon C. Weir, an expert in the field of diabetes research. The Rule 1.132 declaration demonstrates that in Dr. Weir's opinion the Examiner has incorrectly assumed that the cells described in the Peck application are nestin-positive or GLP-1R positive.

The Rule 132 declaration of Dr. Weir describes the current state of the art regarding pancreatic stem cells as complex. That is, **numerous distinct populations of pancreatic stem cells have been identified**.

A population of stem cells that exhibit the phenotype of pancreatic duct cells can serve as precursor cells that differentiate into beta cells (S. Bonner-Weir, M. Taneja, G.C. Weir, K. Tatarkiewicz, K.H. Song, A. Sharma, J.J. O'Neil, In vitro cultivation of human islets from expanded ductal tissue, ***Proc Natl Acad Sci*** U S A 97 (2000) 7999-8004; Exhibit E). Another population of cells that does not exhibit a phenotype of pancreatic duct cells can differentiate toward a beta cell phenotype (R.M. Seaberg, S.R. Smukler, T.J. Kieffer, G. Enikolopov, Z. Asghar, M.B. Wheeler, G. Korbutt, D. Van Der Kooy, Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages, ***Nat Biotechnol*** 22 (2004) 1115-1124; Exhibit F; A. Suzuki, H. Nakauchi, H. Taniguchi, Prospective isolation of multipotent pancreatic progenitors using flow-cytometric cell sorting, ***Diabetes*** 53 (2004) 2143-2152F; Exhibit G). A third population of stem cells has been identified wherein these cells are small and develop some beta cell features (M.Z. Ratajczak, B. Machalinski, W. Wojakowski, J. Ratajczak, M. Kucia, A hypothesis for an embryonic origin of pluripotent Oct-

4(+) stem cells in adult bone marrow and other tissues, ***Leukemia*** 21 (2007) 860-867; Exhibit H). These cells are distinct from the cells described in the Bonner-Weir, Seaberg and Suzuki et al. publications. Finally, other publications describe potential human precursor cells that may be distinct from the cells identified in Peck and described in the above-referenced patent application (L. Ouziel-Yahalom, M. Zalzman, L. Anker-Kitai, S. Knoller, Y. Bar, M. Glandt, K. Herold, S. Efrat, Expansion and redifferentiation of adult human pancreatic islet cells, ***Biochem Biophys Res Commun*** 341 (2006) 291-298; Exhibit I; M.C. Gershengorn, A.A. Hardikar, C. Wei, E. Geras-Raaka, B. Marcus-Samuels, B.M. Raaka, Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells, ***Science*** 306 (2004) 2261-2264; Exhibit J.)

Dr. Weir asserts that given that there are clearly **numerous populations of pancreatic stem cells**, it is his opinion that without additional experimentation one cannot conclude that the cells described in the Peck publication are either nestin-positive or GLP-1R positive. That is, one cannot conclude that the cells described in the Peck et al. publication are "obviously" nestin and/or GLP-1R positive.

Dr. Weir also asserts that it is his opinion that as of the earliest filing date of the instant application (December 6, 1999) one of skill in the art would neither have predicted nor expected the existence of a nestin and/or GLP-1R positive pancreatic stem cell. As of December 6, 1999 the concept of nestin was extremely novel. In addition, the first demonstration of the role of GLP in neogenesis was not until the December 1999 publication from Dr. Habener's laboratory. In view of the unexpected nature of the claimed subject matter of the instant application, Applicants submit that the claimed subject matter is clearly not obvious in view of the Peck et al. application.

In view of all of the above, Applicants submit that claims 39-41, 43, 74, 77-79, and 85-111 are patentable over the WO 97/15310 application. Applicants respectfully request reconsideration and withdrawal of the 35 U.S.C. §103(a) rejection.

Rejection of Claims 74 and 109-111 under 35 U.S.C. §102(b)

Claims 74 and 109-111 are rejected under 35 U.S.C. §102(b) for allegedly being anticipated by WO 97/15310.

Applicants have discussed the '704 patent of Roberts et al. above. Applicants respectfully submit that it is impossible to conclude that the instant claims to "an isolated nestin-positive, GLP-1R positive or nestin and GLP-1R positive human pancreatic or liver stem cells" lack novelty over the '310 application while claim 1 of the '704 patent to "a substantially pure population of human pancreatic progenitor cells, wherein said population of pancreatic progenitor cells will differentiate into acinar, ductal and islet cells" does not.

In view of the above, Applicants request reconsideration and withdrawal of the rejection.

CONCLUSION

Applicants submit that in view of all of the above, all claims are allowable as written and respectfully request early favorable action by the Examiner.

Respectfully submitted,

Date: July 11, 2007

Name: Elizabeth N. Spar
Registration No.: 45,123
Customer No.: 29933
Edwards Angell Palmer & Dodge LLP
P.O. Box 55874
Boston, MA 02110
Tel: 617-239-0100

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Habener et al.	Examiner:	M.A. Belyavskyi
Serial No.:	09/963,875		
Filed:	September 26, 2001	Group Art Unit:	1644
Titled:	Stem Cells of the Islets of Langerhans and Their Use In Treating Diabetes Mellitus	Conf. No.:	9674

DECLARATION UNDER 37 CFR 1.132 BY JOEL F. HABENER, M.D.

I declare:

1. I, Joel F. Habener hold an M.D. degree from the University of California, Los Angeles. I received my M.D. degree in 1965. My current position is Associate Physician at the Massachusetts General Hospital and Professor of Medicine at Harvard Medical School. I have held the position of Associate Physician since 1989. I have held the position of Professor of Medicine at Harvard Medical School since 1989. Previously, I held the position of Associate Professor of Medicine at Harvard Medical School from 1975-1988 and also held the position of Howard Hughes Investigator from 1976-2006. I am an inventor of the above-referenced patent application.

2. I have read the Office Action dated November 16, 2006, filed in the above-referenced patent application and understand that the Examiner has rejected claims 39-41, 43, 77-79, and 85-107 for alleged obviousness over WO 97/15310.

3. Exhibit C, attached, is a JDRF Industry Grant Review Summary Statement dated May 27, 2003 which was written in response to a JDRF grant based on research performed in my

laboratory characterizing pancreatic stem cells. The grant application was prepared and submitted by Viacell Inc., and describes the generation of human beta cells from endogenous pancreatic stems cells and the testing of these cells in transplant models of diabetic mice and primates.

4. The Grant Review Summary Statement was prepared by a panel of experts in the field of diabetes and represents a very recent state of the art critique by those of skill in the art. The Statement evidences a disbelief in the existence of nestin-positive pancreatic stem cells. The reviewers make the following statements that demonstrate clearly the reviewers' disbelief regarding a population of isolated nestin-positive pancreatic stem cells:

- "The reviewers felt that overall the submission of the application is largely premature due to a fundamental lack of supporting preliminary data that is required to support the **novel and somewhat controversial hypothesis presented**."
- "This proposal is based on works performed in the laboratory of Dr. Habener. While the work published by Dr. Habener is interesting, it is **not yet fully accepted by the scientific community**. For example, works performed by the group of Helena Edlund in Sweden or Luc Bouwens in Belgium strongly suggest that nestin-positive cells present in the pancreas are not pancreatic stem cells."
- "If human pancreatic stems cells could be used as a starting source to generate human beta cells, **this would be a dream**."
- "The major problem with this study is that there are at least two publications last year...which very thoroughly have investigated this working hypothesis. Both of them concluded from their data, quite in contrast to the studies by Habener et al. that they call for caution in trying to generate beta cells from nestin-positive cells."
- "The proposal addresses an important issue but for the reasons given above one has to be quite **skeptical** as regards the scientific basis for the study."

5. The review thus establishes that a panel of those skilled in the art did not reasonably expect that a population of nestin-positive stem cells was present and could be isolated from the

pancreas.

6. Exhibit D, attached, is correspondence for the scientific journal Diabetes wherein a manuscript that I prepared and submitted for publication, describing nestin-positive pancreatic stem cells was rejected three times. A panel of expert reviewers evidenced their disbelief in the existence of a nestin-positive pancreatic stem cells.

7. As evidenced by Exhibits C and D presented herein, the results that are presented in the above-referenced patent application with respect to an isolated nestin-positive pancreatic stem cell were unexpected and surprising.

I hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

June 27, 2007
Date

Joel F. Habener

Atty. Docket No.: 203284/1235 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Habener et al.	Examiner:	Belyavskyi, M.A.
Serial No.:	09/963,875		
Filed:	September 26, 2001	Group Art Unit:	1644
Titled:	Protein Production System		
		Conf. No.:	9674

DECLARATION UNDER 37 CFR 1.132 BY, GORDON C. WEIR, M.D.

I Dr. Gordon C. Weir declare:

1. I hold an M.D degree in Medicine from Harvard Medical School. I received my M.D. degree in 1967. My current position is Professor of Medicine at Harvard Medical School and Head of the Section on Islet Transplantation and Cell Biology at Joslin Diabetes Center. I have held these positions since 1990.

2. I am not an inventor of, nor do I have a financial interest in, the above-referenced patent application or its owner.

3. Since 1971, I have performed scientific research in the field of diabetes. In particular, I have extensive experience with the islets of Langerhans. My research has been devoted to determining 1) methods of improving the function of these cells function better; and methods of replenishing these cells through transplantation or stimulation of the growth of these cells in the pancreas of individual diagnosed with diabetes. The work of my colleagues and I have provided

important knowledge to this field. Relevant to this patent issue, my colleagues and I have been deeply involved in the challenge of making new beta cells *in vitro*. We have ourselves made important contributions. We have also followed carefully the work of Peck and Habener. I have over 270 publications relating to the topic of diabetes. I have lectured on the topic of diabetes and the islets of Langerhans at numerous invited seminars. I have reviewed manuscripts presenting data describing diabetes and the islets of Langerhans. I have also received research grants and fellowships both from government and private agencies to fund my investigations of islet transplantation and cell biology. A copy of my curriculum vitae is attached (Exhibit K). I believe that I am an expert in the fields of islet transplantation and cell biology.

4. I understand that the Examiner of the above-referenced patent application has rejected claims 39-41, 43, 77-79 and 85-107 of the patent application for allegedly being obvious in view of Peck et al. WO 97/15310.

5. I have read Peck et al. WO 97/15310.

6. I understand that it is the Examiner's opinion that the Peck et al. reference **does not specifically teach** an isolated nestin-positive or GLP-1R positive human pancreatic or liver stem cell that is not a neural cell. I also understand that the Examiner asserts that the cells described in the Peck et al. publication are "obviously" nestin-positive or GLP-1R positive since the cell population taught in Peck is identical to that claimed in the instant application.

7. It is my opinion that the current state of the art regarding pancreatic stem cells is that multiple types of pancreatic stem cells have been recognized. That is, there is clearly more than one population of pancreatic stem cells.

8. A population of stem cells that exhibit the phenotype of pancreatic duct cells can serve as precursor cells that differentiate into beta cells (S. Bonner-Weir, M. Taneja, G.C. Weir, K. Tatarkiewicz, K.H. Song, A. Sharma, J.J. O'Neil, In vitro cultivation of human islets from expanded ductal tissue, ***Proc Natl Acad Sci*** U S A 97 (2000) 7999-8004; Exhibit E). Another population of cells that does not exhibit a phenotype of pancreatic duct cells can differentiate toward a beta cell phenotype (R.M. Seaberg, S.R. Smukler, T.J. Kieffer, G. Enikolopov, Z. Asghar, M.B. Wheeler, G. Korbutt, D. Van Der Kooy, Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages, ***Nat Biotechnol*** 22 (2004) 1115-1124; Exhibit F; A. Suzuki, H. Nakauchi, H. Taniguchi, Prospective isolation of multipotent pancreatic progenitors using flow-cytometric cell sorting, ***Diabetes*** 53 (2004) 2143-2152F; Exhibit G). A third population of stem cells has been identified wherein these cells are small and develop some beta cell features (M.Z. Ratajczak, B. Machalinski, W. Wojakowski, J. Ratajczak, M. Kucia, A hypothesis for an embryonic origin of pluripotent Oct-4(+) stem cells in adult bone marrow and other tissues, ***Leukemia*** 21 (2007) 860-867; Exhibit H). These cells are distinct from the cells described in the Bonner-Weir, Seaberg and Suzuki et al. publications. Finally, other publications describe potential human precursor cells that may be distinct from the cells identified in Peck and described in the above-referenced patent application (L. Ouziel-Yahalom, M. Zalzman, L. Anker-Kitai, S. Knoller, Y. Bar, M. Glandt, K. Herold, S. Efrat, Expansion and redifferentiation of adult human pancreatic islet cells, ***Biochem Biophys Res Commun*** 341 (2006) 291-298; Exhibit I; M.C. Gershengorn, A.A. Hardikar, C. Wei, E. Geras-Raaka, B. Marcus-Samuels, B.M. Raaka, Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells, ***Science*** 306 (2004) 2261-2264; Exhibit J.)

9. Given that there are clearly **numerous populations of pancreatic stem cells**, it is my opinion that without additional experimentation one cannot conclude that the cells described in the Peck publication are either nestin-positive and/or GLP-1R positive. That is, one cannot

conclude that the cells described in the Peck et al. publication are "obviously" nestin and/or GLP-1R positive.

10. It is also my opinion that as of the earliest filing date of the instant application (December 6, 1999) one of skill in the art would neither have predicted nor expected the existence of a nestin and/or GLP-1R positive pancreatic stem cell. As of December 6, 1999 the concept of nestin was extremely novel. In addition, the first demonstration of the role of GLP in neogenesis was not until the December 1999 publication from Dr. Habener's laboratory. In view of the **unexpected** nature of the claimed subject matter of the instant application, it is my opinion that the claimed subject matter is clearly not obvious in view of the Peck et al. application.

11. In conclusion, it is my opinion that in view of the above, the cells described in the Peck publication are not obviously nestin-positive or GLP-1R positive human pancreatic stem cells.

12. I have received a consulting fee in relationship to the above-referenced patent application.

I hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

7/11/07
Date

Gordon C. Weir

US006436704B1

(12) **United States Patent**
Roberts et al.

(10) **Patent No.: US 6,436,704 B1**
(45) **Date of Patent: Aug. 20, 2002**

(54) **HUMAN PANCREATIC EPITHELIAL PROGENITOR CELLS AND METHODS OF ISOLATION AND USE THEREOF**

(75) Inventors: **Penelope E. Roberts; Jennie Powell Mather**, both of Millbrae, CA (US)

(73) Assignee: **Raven Biotechnologies, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/546,577**

(22) Filed: **Apr. 10, 2000**

(51) **Int. Cl.**[7] **C12N 5/00**; C12N 5/02; C12N 5/06; C12N 5/08

(52) **U.S. Cl.** **435/366**; 435/325; 435/363; 435/378; 435/383; 435/405

(58) **Field of Search** 424/93.7, 93.1; 435/325, 378, 1.1, 363, 405, 383; 800/8; 514/44

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,587,309	A	12/1996	Rubin et al.	
5,834,308	A	11/1998	Peck et al.	
5,888,705	A	3/1999	Rubin et al.	
6,001,647	A *	12/1999	Peck et al.	435/325
6,004,528	A	12/1999	Bergstein	

FOREIGN PATENT DOCUMENTS

EP	0 363 125 A *	4/1990	C12N/5/00
WO	WO 95/29988 A1	11/1995	
WO	WO 97/15310	5/1997	
WO	WO 00/47720 A2	8/2000	
WO	WO 01/42789 A1	6/2001	

OTHER PUBLICATIONS

Beattie, G.M. et al., (1994) "Acid β–galactosidase: a developmentally regulated marker of endocrine cell precursors in the human fetal pancreas" *Journal of Clinical Endocrinology and Methabolism* 78(5):1232–1240.

Hua, Y. and Zhao–Guang, W. (1989) "Morphological and functional studies on microencapsulated human fetal pancreatic tissue" *Chinese Medical Journal* 102(10):786–790.

Hunziker, E. and Stein, M. (2000) "Nestin–expressing cells in the pancreatic islets of langerhans" *Biochemical and Biophysical Research Communications* 271:116–119.

Kritzik, M.R. et al., (1999) "PDX–1 and Msx–2 expression in the regenerating and developing pancreas" *Journal of Endocrinology* 163:523–530.

Kritzik, M.R. et al., (2000) "Expression of ErbB receptors during prancreatic islet development and regrowth" *Journal of Endocrinology* 165:67–77.

Miettinen, P.J. et al., (1993) "Insulin–like growth factor–II and transforming growth factor–α in developing human fetal pancreatic islets" *Journal of Endocrinology* 138:127–136.

Stephan, Jean–Philippe et al., (Dec. 1997). "Characterization of Cell Surface Proteins Using Antibodies Raised to Antigens From Pancreatic Cell Lines," Abstract 1905–presentation materials:17 pages.

(List continued on next page.)

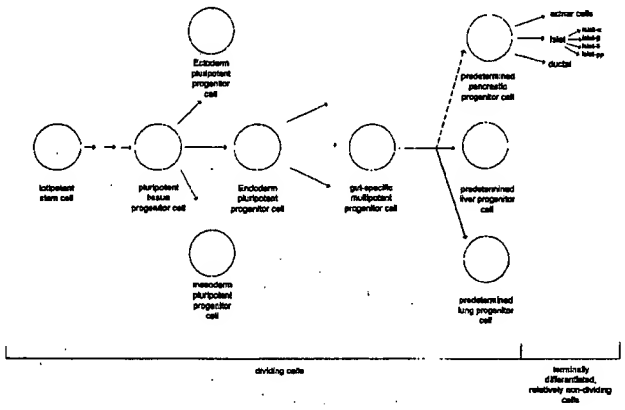
Primary Examiner—Deborah Crouch
Assistant Examiner—Joseph T. Woitach
(74) *Attorney, Agent, or Firm*—Morrison & Foerster LLP

(57) **ABSTRACT**

The invention discloses a substantially pure population of human pancreatic progenitor cells and methods of isolating and culturing the pancreatic progenitor cells. By carefully manipulating the microenvironment of the pancreatic progenitor cells, multiple passages are attainable wherein the pancreatic progenitor cells do not senesce and furthermore, are capable of becoming functional exocrine or endocrine cells. In addition, several methods of use of human pancreatic progenitor cells are disclosed herein.

13 Claims, 8 Drawing Sheets

(7 of 8 Drawing Sheet(s) Filed in Color)



OTHER PUBLICATIONS

Barnes et al., (1980). "Methods for growth of cultured cells in serum-free medium" *Anal. Biochem.* 102:255–270.

Buck et al., (1982). "Monoclonal antibodies specific for cell culture mycoplasmas" In Vitro 18(4):377–381.

Freshney, R.I., (ed), (1987). *Animal Cell Culture* pp.vii–xii. (Table of Contents).

Ganong, William F., (1991). "Regulation of gastrointestinal function" in *Review of Medical Physiology*, fifteenth edition, Appleton and Lange, (eds), Chapter 26, pp. 448–447.

Ham et al., (1979). "Media and growth requirements" *Meth. Enzy.* 58:44–93.

Harlow and Lane, (eds), (1988). *Antibodies, A Laboratory Manual* pp. iii–ix. (Table of Contents).

Köhler et al., (1975). "Continuous cultures of fused cells secreting antibody of predefined specificity" *Nature* 256:495–497.

Mather, Jennie P. and Roberts, Penelope E., (1998). *Introduction to Cell and Tissue Culture* Plenum Press, New York pp. xi–xiv. (Table of Contents).

Olumi et al., (1999). "Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium" *Cancer Research* 59:5002–5011.

Stephan et al., (1999)."Selective cloning of cell surface proteins involved in organ development: Epithelial glycoprotein is involved in normal epithelial differentiation" *Endocrinology* 140:5841–5854.

Levine et al. Transplantation Proceedings 27(6):3410, Dec. 1995.*

Mathers et al. Experientia 41(1):116–8, Jan. 1985.*

Kolar et al. Pancreas 15(3):265–71, Oct. 1997.*

Levine et al. Cell Transplantation 3(4):307–13, Jul. 1994.*

Dabeva et al. Proceedings of the National Academy of Science 94:7356–61, Jul. 1997.*

Mandel, T.E. Journal of Molecular Medicine 77:155–160, Jan. 1999.*

Taniguchi et al. Journal of Surgical Research 70:41–45, 1997.*

* cited by examiner

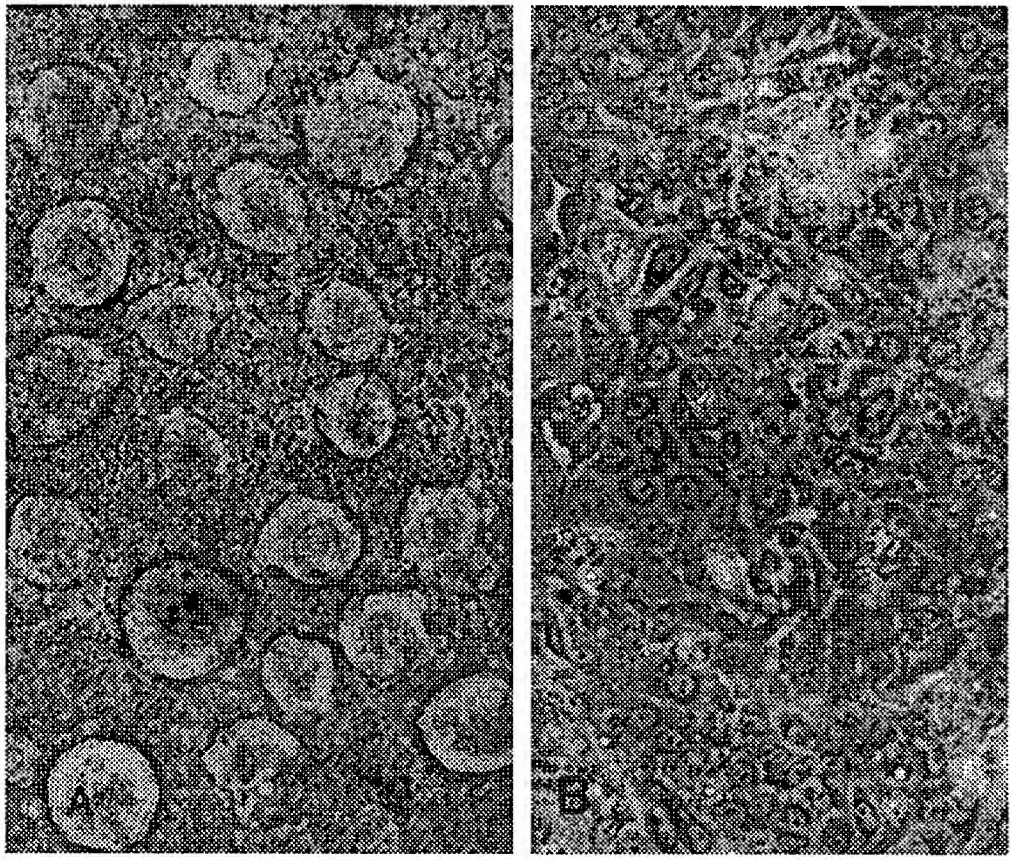


Figure 1

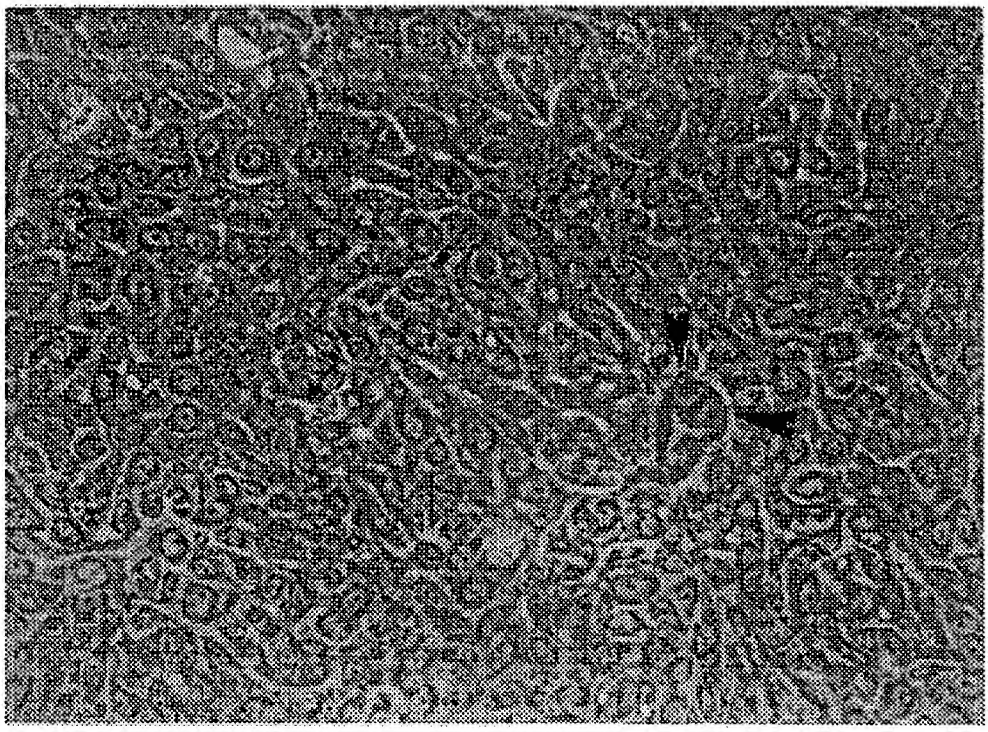


Figure 2:

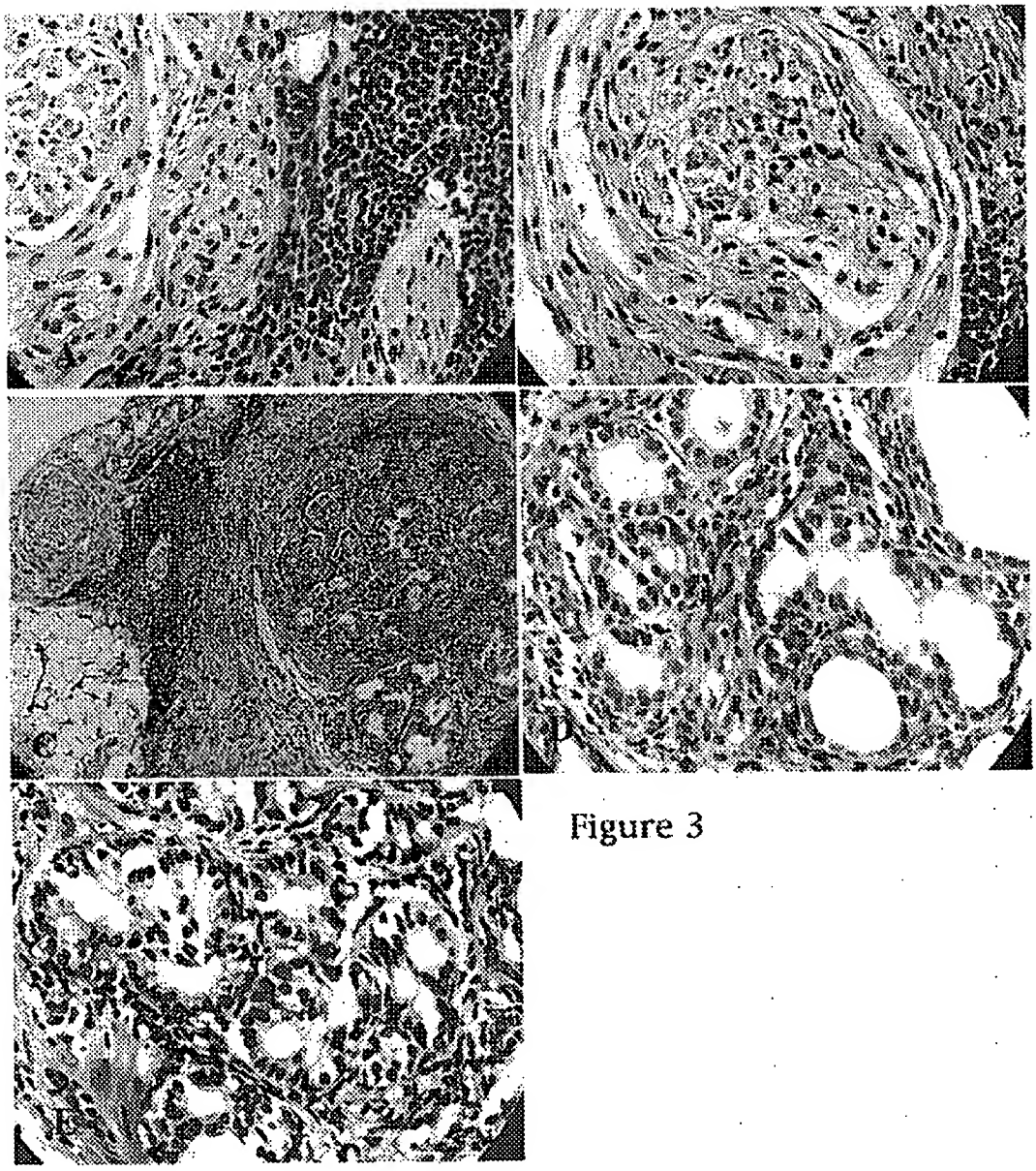


Figure 3

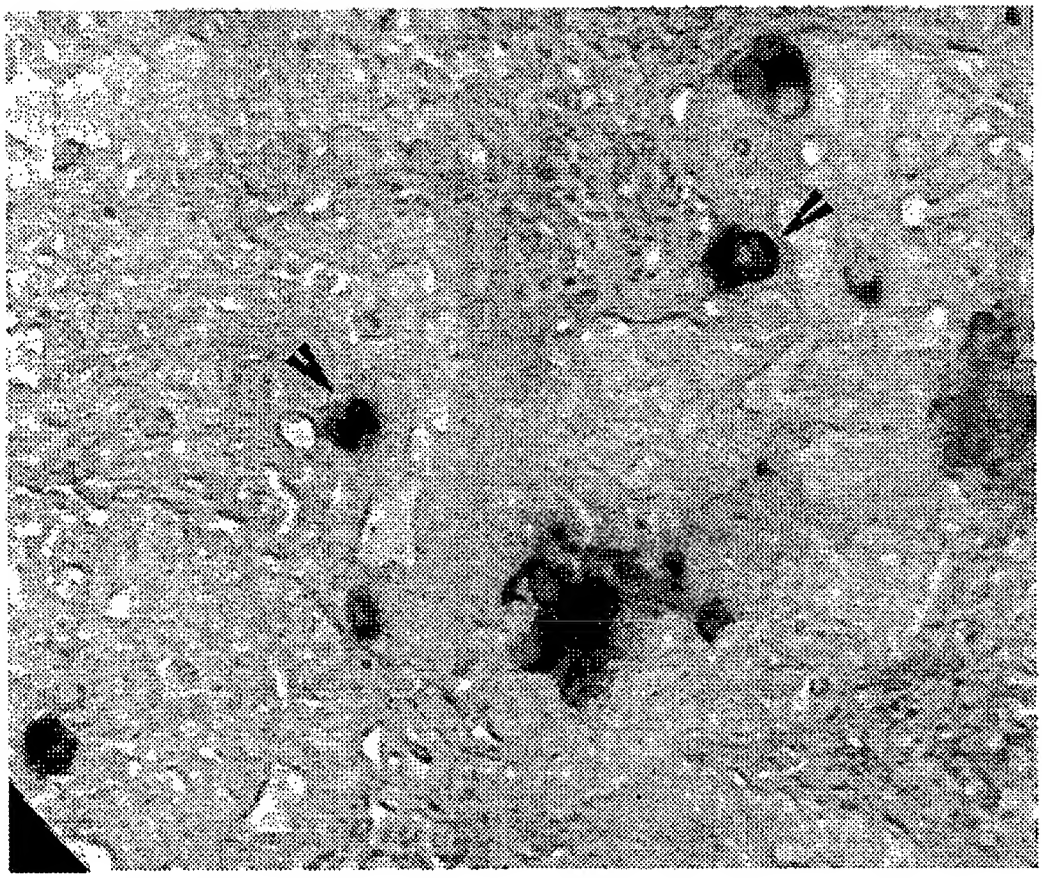


Figure 4

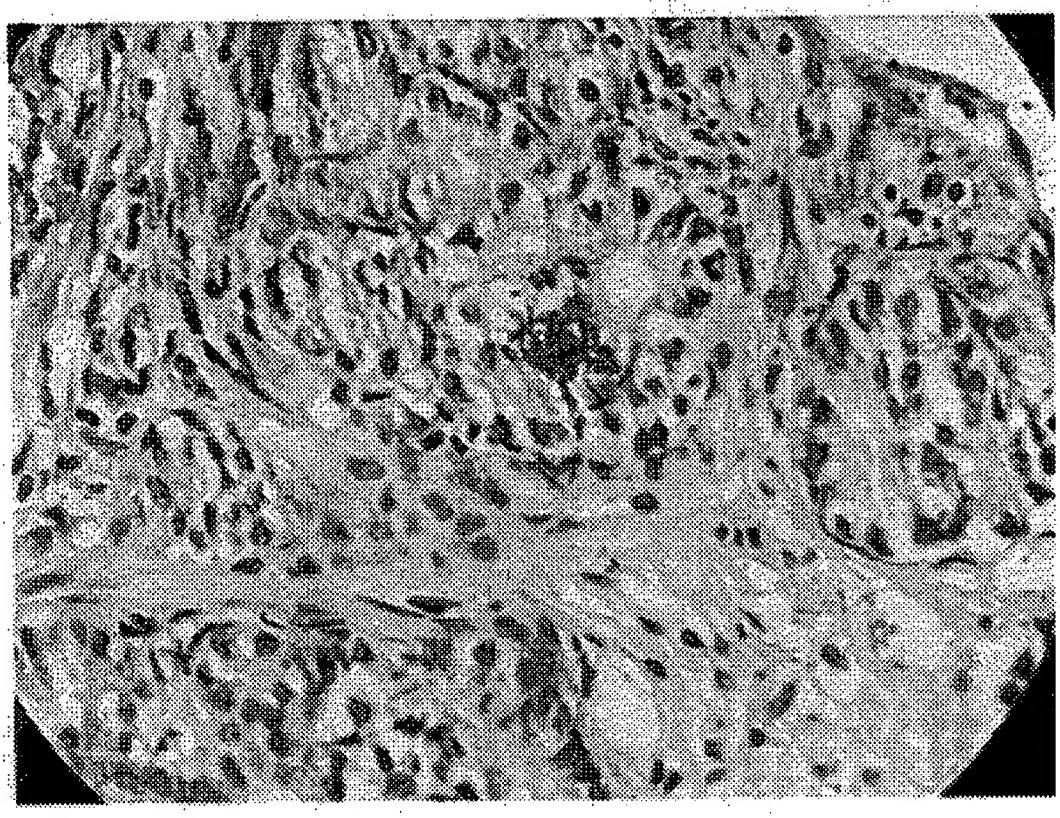


Figure 5

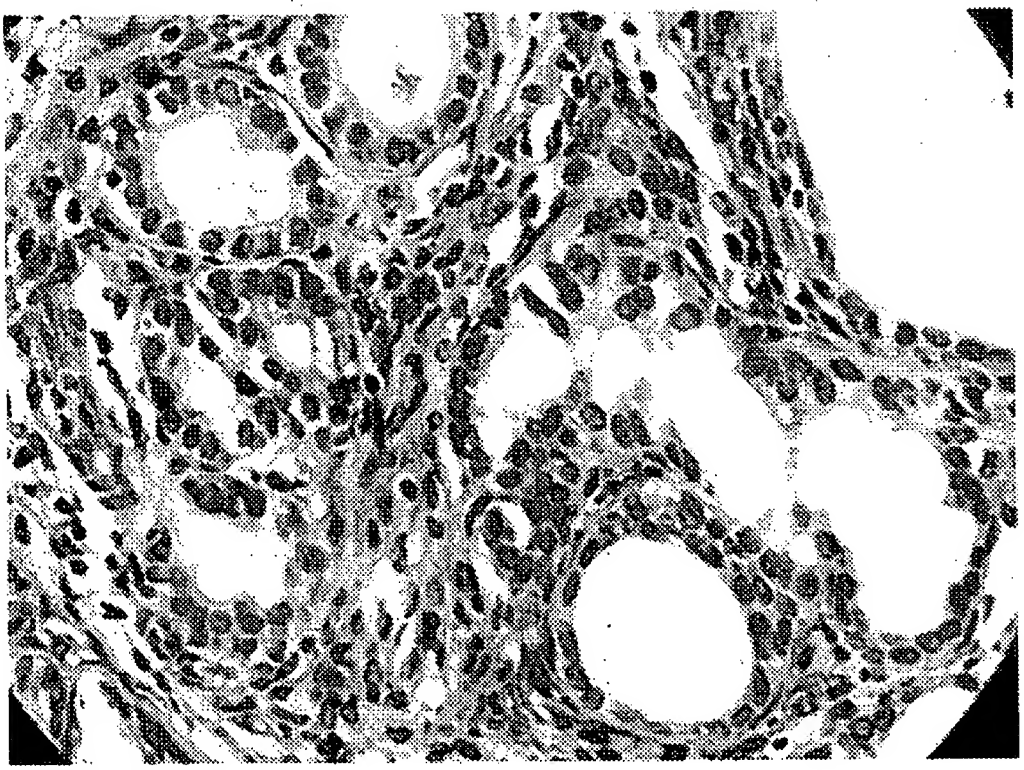


Figure 6

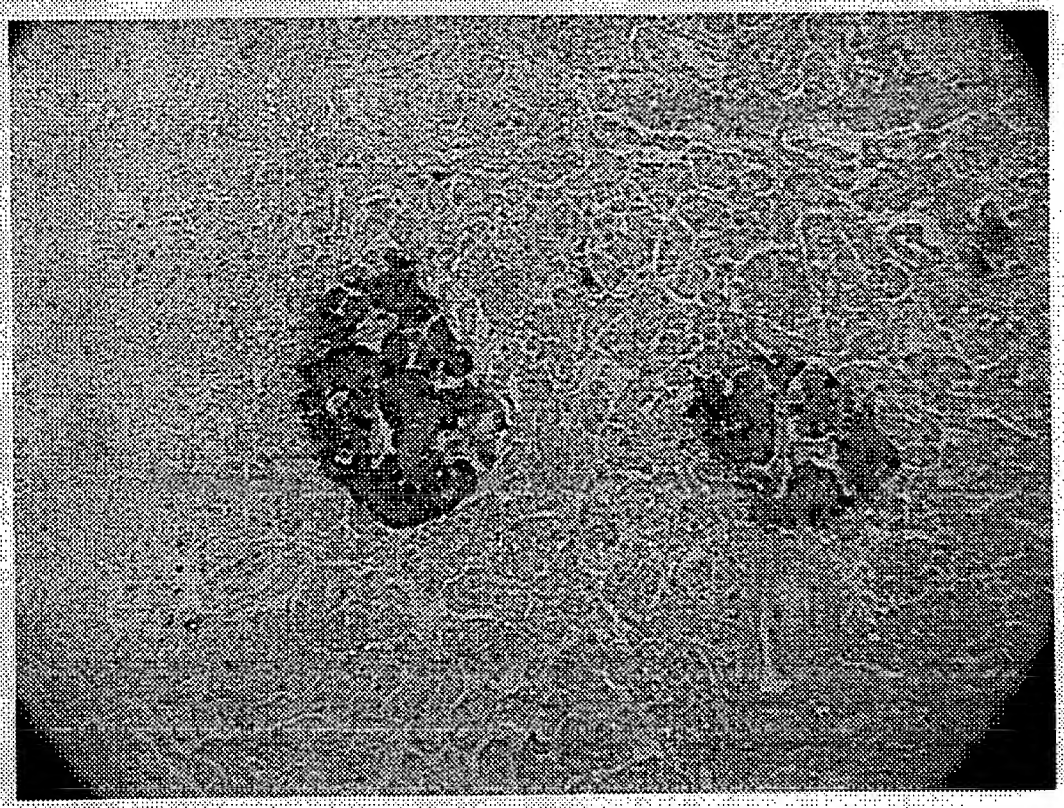


Figure 7

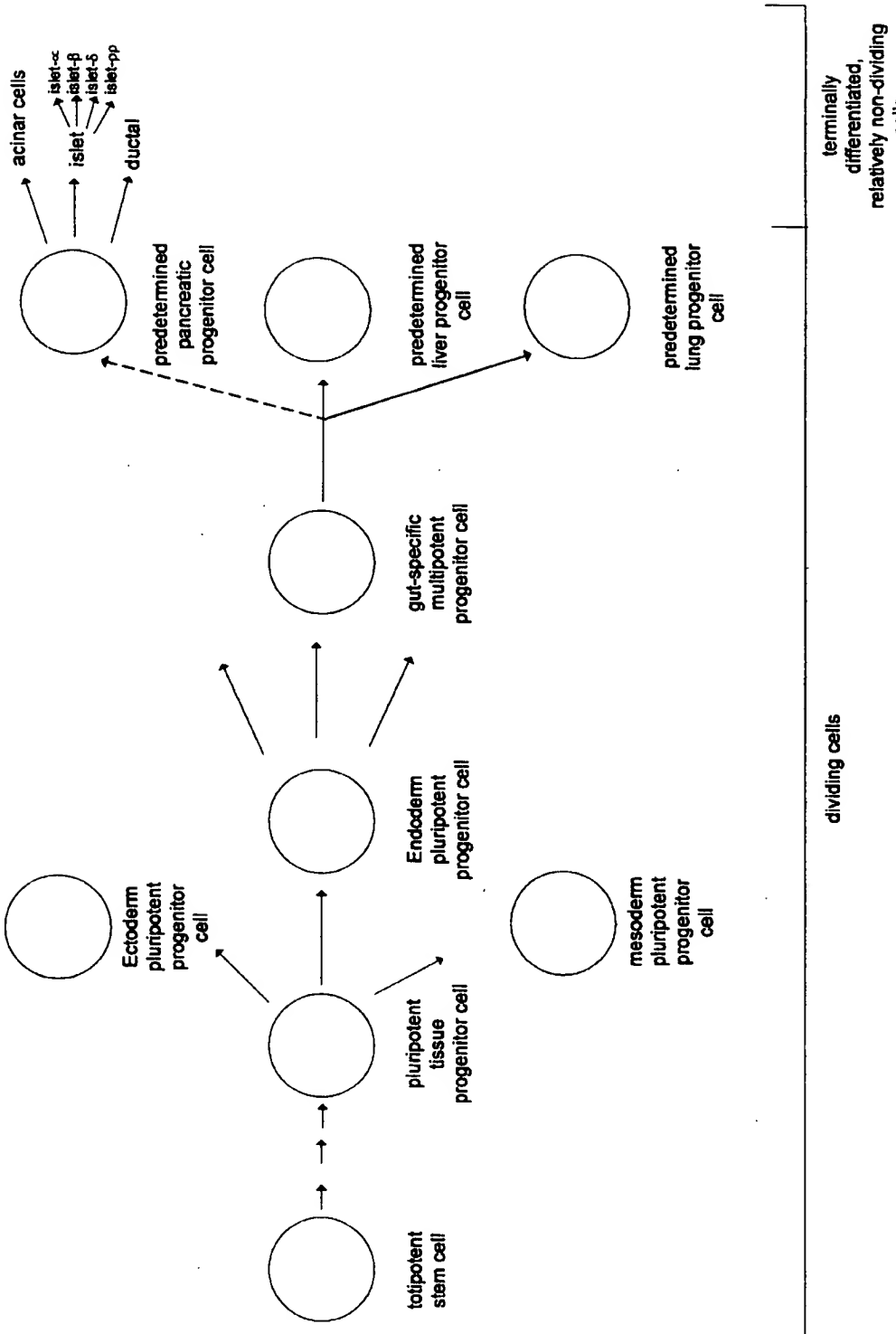

totipotent stem cell
pluripotent tissue progenitor cell
Ectoderm pluripotent progenitor cell
Endoderm pluripotent progenitor cell
mesoderm pluripotent progenitor cell
gut-specific multipotent progenitor cell
predetermined pancreatic progenitor cell
predetermined liver progenitor cell
predetermined lung progenitor cell
acinar cells
islet
islet-α
islet-β
islet-δ
islet-pp
ductal
dividing cells
terminally differentiated, relatively non-dividing cells

FIGURE 8

HUMAN PANCREATIC EPITHELIAL PROGENITOR CELLS AND METHODS OF ISOLATION AND USE THEREOF

TECHNICAL FIELD

This invention is in the field of developmental biology and cell biology. Specifically, this invention relates to a population of pancreatic epithelial progenitor cells that are capable of differentiating into functional endocrine and exocrine cells, methods of isolating the pancreatic epithelial progenitor cells, characterization of pancreatic epithelial progenitor cells, and uses of the pancreatic epithelial progenitor cells.

BACKGROUND ART

Stem cell and progenitor cell isolation and characterization are the subjects of intense research because of the great potential of such cells. The totipotent stem cells, which have the capacity to become any type of cell in a human body, give rise to progenitor cells more differentiated than the totipotent cell. One of these types of progenitor cells is the predetermined pancreatic epithelial progenitor cell. The pancreatic epithelial progenitor cells have the ability to become different types of pancreatic epithelial cells. The different types of pancreatic epithelial cells include acinar cells, islet cells, and ductal cells. Acinar cells are generally found near the head of the pancreas and contain zymogen granules which are readily visible by electron microscopy. Acinar cells perform exocrine functions by discharging alkaline digestive juices into the small intestine. Approximately 1500 mL of pancreatic juices are secreted per day and include enzymes needed to break up lipids and proteins. Ganong, William F. Review of Medical Physiology, Chapter 26 "Regulation of Gastrointestinal Function", Fifteenth Edition, Appleton and Lange (1991). There are four types of islet cells, also known as islets of Langerhans, islet-α, islet-β, islet-δ, and islet-PP. Islet-α cells secrete glucagon which promotes gluconeogenesis, i.e. breakdown of energy reserves to generate more circulating glucose. Islet-β cells secrete insulin which promotes storage of circulating glucose into accessible energy resources. In type I diabetes mellitus, otherwise known as juvenile diabetes, it is thought that autoimmune attacks on islet-β cells cause defective islet-β cell function, thereby causing a lack of insulin to reduce the levels of circulating glucose. Islet-δ cells secrete somatostatin which regulates the secretion of glucagon and insulin. The fourth islet cell type islet-PP (pancreatic polypeptide) does not yet have a known function within the pancreas. Another type of sub-pancreatic cell is the ductal cell. These cells line the ducts that connect different parts of the pancreas.

Isolation of pancreatic epithelial progenitor cells, as with other types of progenitor cells, is difficult because of the ephemeral nature of progenitor cells. Manipulation of progenitor cells required for isolation may disturb the fragile progenitor status of these cells and may cause them to differentiate. Contact with growth factors or substrates may also induce a pancreatic progenitor cell to begin differentiating into exocrine or endocrine cells. Research in the area of pancreatic cells has resulted in the establishment of several pancreatic epithelial cell lines derived from rats. Stephan, J. et. al. Endocrinology 140:5841–5854, (1999). Other research includes the isolation of human adult pancreatic cells and the induction of these pancreatic cells to proliferate into islet-β-like structures with hepatocyte growth factor/scatter factor (HGF/SF). Jeffrey et. al. U.S. Pat. No. 5,888,705. Other research work involves inducing growth of islet cells from adult pancreatic cells by culturing first in serum-containing, low-glucose medium and then switching to medium with higher serum and glucose content. WO 9715310. Still other research in the area of pancreatic progenitor cells includes isolating progenitor cells from pre-diabetic adults and culturing in a serum-containing, pre-defined media that promotes the growth of functional islet cells. U.S. Pat. No. 5,834,308. However, all of these "progenitor" cells give rise only to islet cells. Pancreatic cells of the aforementioned research do not have the capacity to differentiate into both endocrine and exocrine cell types. It seems likely that the pancreatic cells of the aforementioned research are further committed down the differentiation pathway of pancreatic progenitor cells and therefore are different types of pancreatic cells than the human pancreatic progenitor cells of this invention. Furthermore, culturing conditions used in the aforementioned research wherein serum is used to supplement media may have adverse consequences. Serum, the fluid portion of blood after blood has been allowed to clot, contains many biomolecules such as albumin and α, β, -globulins. In vivo, cells are not normally exposed to an equivalent of serum unless tissue injury was involved. Therefore, culturing pancreatic cells in serum may not accurately reflect the physiological parameters within which pancreatic cells exist in vivo.

The ideal population of pancreatic progenitor cells should be able to differentiate into exocrine (i.e. acinar) cells, endocrine (i.e. islet-α, islet-β, islet-δ, and islet-PP) cells as well as ductal cells. Such a population of pancreatic progenitor cells may be useful in clinical settings, for example to treat certain types of diabetes or to treat functionally defective pancreatic cells by transplantation of pancreatic progenitor cells that can differentiate into functional pancreatic cells. Accordingly, there is a need for a population of pancreatic progenitor cells and methods of isolating and culturing the pancreatic progenitor cells such that the differentiation potential of the pancreatic progenitor cells is retained while permitting proliferation and avoiding senescence of these cells. The pancreatic progenitor cells and methods of isolating and culturing these pancreatic progenitor cells disclosed herein satisfies these needs and also provides related advantages.

DISCLOSURE OF THE INVENTION

This invention is related to the field of developmental and cell biology. In one aspect, the invention relates to a population of substantially pure human pancreatic epithelial progenitor cells which have a pluripotent capability to differentiate into functional exocrine or endocrine pancreatic cells.

In another aspect of this invention, the invention relates to methods of isolating a population of substantially pure human pancreatic epithelial progenitor cells which have the pluripotent capability to differentiate into functional exocrine or endocrine pancreatic cells.

In yet another aspect of this invention, the invention relates to methods of maintaining a population of substantially pure human pancreatic epithelial progenitor cells which have the pluripotent capability to differentiate into functional exocrine or endocrine pancreatic epithelial cells and maintaining or culturing these pancreatic progenitor cells such that the cells retain their pluripotent capacity while avoiding senescence.

In still another aspect of this invention, the invention relates to methods of providing a source of immunogen and

the uses of a substantially pure population of pancreatic progenitor cells as an immunogen.

In still another aspect of this invention, the invention relates to methods of generating a human pancreatic tissue model using a substantially pure population of pancreatic progenitor cells as a source of pancreatic cells and introducing the pancreatic progenitor cells into a non-human, mammalian recipient.

In another aspect of this invention, the invention relates to methods of providing cell therapy whereby a substantially pure population of human pancreatic progenitor cells are introduced into a recipient.

In another aspect of this invention, the invention relates to methods of providing pharmaceutical drug development wherein a substantially pure population of human pancreatic progenitor cells are used as a source of pancreatic biological components in which one or more of these pancreatic biological components are the targets of the drugs that are being developed.

In another aspect of this invention, the invention relates to methods of providing bioassay development wherein a substantially pure population of human pancreatic progenitor cells are used as a source of nucleic acids or proteins and wherein these nucleic acids or proteins are used as one or more principal components in a bioassay or the development of a bioassay.

BRIEF DESCRIPTION OF THE DRAWINGS

This patent or application file contains at least one drawing executed in color. Copies of this patent or patent application with color drawing(s) will be provided by the U.S. Patent and Trademark Office upon request and payment of the necessary fee.

FIG. **1** shows human pancreatic ductal epithelial cells grown in two different types of media. FIG. **1A** (left) shows pancreatic epithelial cells grown in CMRL 1066 medium with fibronectin coating on the plate. The large, rounded cells are pancreatic epithelial cells. FIG. **1B** (right) shows pancreatic epithelial cells grown in F 12/DMEM medium. The pancreatic epithelial cells have flatten out to form a monolayer.

FIG. **2** shows human pancreatic epithelial cells grown on collagen-coated plates after three passages. The arrows denote dividing cells.

FIG. **3** shows results of staining of tissue recombinant grafts placed under the fat pad of an immunodeficient mouse. FIG. **3A** shows islet formation in the tissue recombinant graft at a magnification of 20x. FIG. **3B** shows islet formation in the tissue recombinant graft at a magnification of 60x. FIG. **3C** shows formation of islet, duct, and acinar tissue within the tissue recombinant graft. FIG. **3D** shows ductal formation in the tissue recombinant graft. FIG. **3E** shows formation of clusters (or aggregates) of acinar cells in the tissue recombinant graft.

FIG. **4** shows the results of staining for glucagon (blue) and insulin (brown) in the tissue recombinant graft which was placed under the kidney capsule of an immunodeficient mouse.

FIG. **5** shows the results of staining for insulin (brown) in the tissue recombinant graft which was placed under the kidney capsule of an immunodeficient mouse.

FIG. **6** shows ductal formation in the tissue recombinant which was placed under the fat pad of an immunodeficient mouse.

FIG. **7** shows the results of staining for glucagon (blue) and insulin (brown) in paraffin-embedded tissue section from a tissue graft which was placed under the kidney capsule of an immunodeficient mouse.

FIG. **8** is a schematic depiction of the development of a pancreatic cells from a totipotent stem cell. The dotted line indicates the stage of differentiation at which the human pancreatic progenitor cell of this invention resides.

MODES FOR CARRYING OUT THE INVENTION

The following detailed description of the invention is provided to aid those skilled in the art in practicing the present invention. This detailed description should not be construed to limit the present invention, as modifications of the embodiments disclosed herein may be made by those of ordinary skill in the art without departing from the spirit and scope of the present invention. Throughout this disclosure, various publications, patents, and published patent specifications are referenced by citation. The disclosure of these publications, patents, and published patents are hereby incorporated by reference in their entirety into the present disclosure.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art. See, e.g., Sambrook, et al. MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M. J. MacPherson, B. D. Hames and G. R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R. I. Freshney, ed. (1987)).

Definitions

As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

As used in the specification and claims, the terms "pancreatic epithelial progenitor cells" and "pancreatic progenitor cells" are interchangeable and refer to "pancreatic epithelial progenitor cells" and "pancreatic progenitor cells" of human origin.

"Pancreatic epithelial progenitor cells" and "pancreatic progenitor cells" refer to dividing progenitor cells found in the pancreas that have not yet committed to an essentially non-dividing stage of end differentiation. "Pancreatic epithelial progenitor cells" and "pancreatic progenitor cells" are derived ultimately from totipotent cells that give rise to pluripotent, tissue-specific cells. These pluripotent, tissue-specific, dividing progenitor cells can give rise to cells of the endoderm, ectoderm, or mesoderm. Of the endodermal multipotent cells, some differentiate into gut-specific, dividing progenitor cells. Of the gut-specific progenitor cells, some are pre-determined to become pancreatic cells. It is at this stage of development that the population of cells claimed herein resides. More specifically, the population of "pancreatic epithelial progenitor cells" and "pancreatic progenitor cells" disclosed herein is between the stage at which a gut-specific progenitor cell is pre-determined to become a pancreas (or part of a pancreas) and the stage at which a pancreas-specific progenitor cell is committed to becoming a sub-pancreatic type of cell. Pancreas-specific progenitor cells can differentiate into several types of cells: acinar, ductal, and islet-α, islet-β, islet-δ, and islet-PP. One exocrine function of the acinar cells is the secretion of digestive juices

into the intestine. One endocrine function of the islet cells is the secretion of glucagon (islet-α) and insulin (islet-β). The pancreatic progenitor cells of this invention have not differentiated into any of the aforementioned types of sub-pancreatic cells but have the capacity to become any of these cells.

"Sub-pancreatic" refers to cellular infrastructure within the pancreas as a whole organ. Examples of sub-pancreatic cells include, but are not limited to, acinar, ductal, and islet cells.

"Totipotent cell" and "totipotent stem cell" are used interchangeably throughout and refer to a stem cell that has the capacity to become any type of cell in a mammalian body.

"Pluripotent" and "multipotent" are used interchangeably throughout and refer to a stage where a cell can still become one of a plurality of cells but can no longer become any type of cell in the body. "Pluripotent" cells are not referred to as "stem cells" but rather "progenitor cells" because they are progenitors to one or more type of a plurality of cells.

As used herein, "pre-determined pancreatic" refers to a stage of development of a multipotent cell that is beyond the stage of being gut-specific and before the stage of terminally differentiated pancreatic cells (such as acinar, islet, or ductal cells). Cells which are "pre-determined pancreatic" are committed to becoming pancreatic cells but have not begun to develop into terminally differentiated pancreatic cells yet. Different factors cause pre-determined pancreatic cells to begin differentiating. Non-limiting examples include exposure to serum, exposure to insulin growth factor (IGF) or epidermal growth factor (EGF), contact with surrounding tissue, microenvironment of the cells, and cell-cell contact with surrounding tissue. The chain of development begins with a totipotent stem cell which can become any cell in the body. The totipotent stem cell is a true stem cell because of its cellular omnipotency. At any stage beyond the totipotent stem cell, cells become a "pre-determined progenitor" because they have been committed down a pathway that no longer enables the cell to become any kind of cell in the body.

An "antibody" is an immunoglobulin molecule capable of binding an antigen. As used herein, the term encompasses not only intact immunoglobulin molecules, but also anti-idiotypic antibodies, mutants, fragments, fusion proteins, humanized proteins and modifications of the immunoglobulin molecule that comprise an antigen recognition site of the required specificity.

The term "antigen" is a molecule which can include one or more epitopes to which an antibody may bind. An antigen is a substance which can have immunogenic properties, i.e., induce an immune response. Antigens are considered to be a type of immunogen. As used herein, the term "antigen" is intended to mean full length proteins as well as peptide fragments thereof containing or comprising one or a plurality of epitopes.

The terms "surface antigens" and "cell surface antigen" are used interchangeably herein and refer to the plasma membrane components of a cell. These component include, but are not limited to, integral and peripheral membrane proteins, glycoproteins, polysaccharides, lipids, and glycosylphosphatidylinositol (GPI)-linked proteins. An "integral membrane protein" is a transmembrane protein that extends across the lipid bilayer of the plasma membrane of a cell. A typical integral membrane protein consists of at least one membrane spanning segment that generally comprises hydrophobic amino acid residues. Peripheral membrane proteins do not extend into the hydrophobic interior of the lipid bilayer and they are bound to the membrane surface by noncovalent interaction with other membrane proteins. GPI-linked proteins are proteins which are held on the cell surface by a lipid tail which is inserted into the lipid bilayer.

The term "monoclonal antibody" as used herein refers to an antibody composition having a substantially homogeneous antibody population. It is not intended to be limited as regards to the source of the antibody or the manner in which it is made (e.g. by hybridoma or recombinant synthesis). Monoclonal antibodies are highly specific, being directed against a single antigenic site. In contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

"A population of monoclonal antibodies" refers to a plurality of heterogeneous monoclonal antibodies, i.e., individual monoclonal antibodies comprising the population may recognize antigenic determinants distinct from each other.

"Immunogen" refers to any substance that induces an immune response. A substance that is an immunogen is described as being "immunogenic". Induction of immune response includes but is not limited to activation of humoral responses (e.g. producing antibodies) or cellular responses (e.g. priming cytotoxic T cells), inflammatory responses (e.g. recruitment of leukocytes), and secretion of cytokines and lymphokines.

The term "heterologous" as applied to a cell used for immunization or transplantation means that the cell is derived from a genotypically distinct entity from the recipient. For example, a heterologous cell may be derived from a different species or a different individual from the same species as the recipient. An embryonic cell derived from an individual of one species is heterologous to an adult of the same species.

A cell is of "ectodermal", "endodermal" or "mesodomal" origin, if the cell is derived, respectively, from one of the three germ layers—ectoderm, the endoderm, or the mesoderm of an embryo. The ectoderm is the outer layer that produces the cells of the epidermis, and the nervous system. The endoderm is the inner layer that produces the lining of the digestive tube and its associated organs, including but not limited to pancreas and liver. The middle layer, mesoderm, gives rise to several organs (including but not limited to heart, kidney, and gonads), connective tissues (e.g., bone, muscles, tendons), and the blood cells.

The terms "medium", "cell culture medium", and "culture medium" are used interchangeably. The terms refer to the aqueous microenvironment in which the mammalian cells are grown in culture. The medium comprises the physicochemical, nutritional, and hormonal microenvironment.

A cell culture medium is "essentially serum-free" when the percentage by volume of serum in the medium does not mask antigenic sites or antibody binding sites on cell surfaces. The term "essentially serum-free" generally applies when the cell culture medium comprises less than about 50% serum (by volume), preferably less than about 25% serum, even more preferably less than about 5% serum, and most preferably less than about 0.1% serum.

A cell surface is "substantially free of serum biomolecules" when at least about 75% of the pancreatic progenitor cell surfaces, more preferably at least about 90% of the pancreatic progenitor cell surfaces, even more preferably at least about 95% of the pancreatic progenitor cell surfaces, and most preferably at least about 99% of the pancreatic

progenitor cell surfaces do not have serum biomolecules derived from serum binding to the cell surface such that antigenic sites or antibody binding sites are bound or are unavailable for antigenic recognition by an antibody or a portion of an antibody. Cell surface can determined by measuring the cell size, either by microscopy or flow cytometry. For example, synthetic beads of various known sizes are commonly used for calibration in flow cytometry. A small quantity of calibrated bead may be mixed with pancreatic progenitor cells and the resultant population is analyzed by flow cytometry. Pancreatic progenitor cell can then be compared with the size of the calibrated beads. Calculations of cell surface amount can be accomplished since the sizes of the beads are known.

As used herein, a "substantially pure" population of pancreatic progenitor cells is a population of cells that is comprised at least about 85% pancreatic progenitor cells, preferably at least about 90%, and even more preferably about 95% or more.

A "defined medium" and "basal cell-sustaining medium" are used interchangeably herein and refer to a medium comprising nutritional and hormonal requirements necessary for the survival and/or growth of the cells in culture such that the components of the medium are known. Traditionally, the defined medium has been formulated by the addition of nutritional and growth factors necessary for growth and/or survival. Typically, the defined medium provides at least one component from one or more of the following categories: a) all essential amino acids, and usually the basic set of twenty amino acids plus cystine; b) an energy source, usually in the form of a carbohydrate such as glucose; c) vitamins and/or other organic compounds required at low concentrations; d) free fatty acids; and e) trace elements, where trace elements are defined as inorganic compounds or naturally occurring elements that are typically required at very low concentrations, usually in the micromolar range. The defined medium may also optionally be supplemented with one or more components from any of the following categories: a) one or more mitogenic agents; b) salts and buffers as, for example, calcium, magnesium, and phosphate; c) nucleosides and bases such as, for example, adenosine and thymidine, hypoxanthine; and d) protein and tissue hydrolysates.

As used herein, "conditioned media" refers to culture media, free of intact cells, in which pancreatic epithelial progenitor cells have been grown. Pancreatic cells grown in nutrient media may release factors which promote the continued survival, growth, and maintenance of pre-existing state of pre-differentiation of the pancreatic progenitor cells. Conditioned media may be used to reconstitute a cell pellet or added to cells already existing in culture plates. Conditioned media may also be used alone or to supplement nutrient media being used to feed pancreatic cells. Since conditioned media derived from nutrient media and nutrient media, as described herein, is essential serum-free, conditioned media is also essentially serum-free.

"Standard incubation conditions" refers to the physicochemical conditions in an incubator designed for tissue culture in which cells are placed. Generally, the standard incubation conditions are about 37 degrees Celsius and about 5% CO_2 content with humidification. All tissue culture techniques and equipment should be performed under sterile conditions. Tissue culture containers refer to any type of container that may be used for culturing cells. Non-limiting examples include flasks and plates.

A "mitogenic agent" or "growth factor" is a molecule which stimulates mitosis of the mammalian cells. Generally, the mitogenic agent or growth factor enhances survival and proliferation of mammalian cells in cell culture and is a polypeptide. The mitogenic polypeptide can be a "native" or "native sequence" polypeptide (i.e. having the amino acid sequence of a naturally occurring growth factor) regardless of the method by which it is produced (e.g. it can be isolated from an endogenous source of the molecule or produced by synthetic techniques including recombinant techniques), or a variant or mutant thereof (see definition below). Non-limiting examples include activators of one or more members of the erbB receptor family; agents which elevate cAMP levels in the culture medium (e.g. forskolin, cholera toxin, cAMP or analogues thereof); adhesion molecules such as neural cell adhesion molecule (N-CAM), laminin or fibronectin; progesterone; neurotrophic factors such as bone-derived neurotrophic factor (BDNF) and ciliary neuronotrophic factor (CNTF); neurotrophin-3, -4, -5, or -6; platelet-derived growth factor (PDGF); fibroblast growth factor such as acidic FGF (aFGF) and basic FGF (bFGF); vascular endothelial growth factor (VEGF); transforming growth factor (TGF) such as TGF-α and TGF-β; insulin-like growth factors, including IGF-I and IGF-II; hormones such as estrogen, testosterone, thyroid hormone, insulin and any of those mitogens listed in Table 8.2 at pages 138–139 of Mather, J. P. and Roberts, P. E. (1998) "Introduction to Cell and Tissue Culture", Plenum Press, New York.

"Pancreatic progenitor cell aggregates", "pancreatic progenitor cell spheres", and "pancreatic cell clusters" are used interchangeably throughout and refers to a mass of a plurality of pancreatic progenitor cells which can form a three-dimensional structure resembling roughly a sphere.

A "grafting recombinant", as used herein, refers to the combined unit of pancreatic progenitor cell aggregates placed with mesenchymal tissue. Mesenchymal tissue can be of pancreatic or non-pancreatic origin. Mesenchymal tissue can be from a species heterologous to the graft recipient. Mesenchymal tissue can also be from a species heterologous to the source of pancreatic progenitor cells. Grafting recombinants can be incubated on substrate, preferably a soft, biological substrate (e.g. agar) for a period ranging from 1 hour to 72 hours, more preferably between 6 hours to 24 hours, and even more preferably, overnight with an incubation period of about 8 to 16 hours. Olumi A. F., et. al. Cancer Research 59, 5002–5011, (1999).

"Serum", as used herein, refers to the fluid phase of mammalian blood that remains after blood is allowed to clot.

"Serum biomolecules", as used herein, refers to biological compositions found in serum. Examples include, but are not limited to, albumin, α1-globulin, α2-globulin, β-globulin, and δ-globulin. Serum biomolecules can include biological compositions, whole or partial, that are either naturally found in serum or derived from processing and handling of serum.

The terms "mammals" or "mammalian" refer to warm blooded vertebrates which include but are not limited to humans, mice, rats, rabbits, simians, sport animals, and pets.

Isolation and Maintenance of Pancreatic Progenitor Cells

Pancreatic progenitor cells of this invention are isolated from human fetal pancreatic tissue. The age of the fetus is between about week 6 and about week 40, preferably between about week 8 and about week 26, and even more preferably between about week 12 and about week 22. The pancreatic tissue can be identified by gross anatomy, outward appearance, and location within the fetus. Several features of gross anatomy and appearance distinguishing a pancreas are: an elongated lobulated retroperitoneal gland, lack of capsule, and extension from the concavity of the

duodenum of the intestine to the spleen. The pancreas consists of a flattened head or caput within the duodenal concavity, an elongated three-sided body extending transversely across the abdomen, and a tail in contact with the spleen. Once identified, fetal pancreatic tissue is microdissected. The purpose of microdissection is to separate structures containing epithelial cells from connective tissue and non-pancreatic tissue such as fat, membranes, etc. or to separate cells from each other. Non-limiting examples of microdissection include devices that render mechanical shearing forces (i.e. homogenizer, mortar and pestle, blender, etc.), devices that render cuts or tears (i.e. scalpel, syringes, forceps, etc.), or ultrasonic devices. Alternatively, another method of microdissecting fetal pancreatic tissue is the use of enzyme treatment. Various enzyme treatments used to microdissect tissue are well known in the art. One method includes the use of collagenase-dispase to digest partially sheared pancreatic tissue in a buffered medium that will sustain viability of cells isolated from the pancreatic tissue. A concentration of at least about 0.5 mg/ml collagenase-dispase is used, more preferably at least about 1 mg/ml and even more preferably at least about 5 mg/ml. The amount of enzyme will depend on the age of the fetus and how large the pancreatic tissue is. In the preferred embodiment, pancreatic tissue from fetus between about 14 weeks and about 22 weeks is digested with about 5 mg/ml of collagenase-dispase. A wide variety of basal cell-sustaining media that can be used to keep the pH of the liquid in a range that promotes survival of pancreatic progenitor cells and to provide additional volume of liquid within which the enzymatic digestion can occur. Non-limiting examples include F12/DMEM, Ham's F10 (Sigma), CMRL-1066, Minimal essential medium (MEM, Sigma), RPMI-1640 (Sigma), Dulbecco's Modified Eagle's Medium (DMEM, Sigma), and Iscove's Modified Eagle's Medium (IMEM). In addition, any of the basal nutrient media described in Ham and Wallace *Meth. Enz.*, 58:44 (1979), Barnes and Sato *Anal. Biochem.*, 102:255 (1980), or Mather, J. P. and Roberts, P. E. "Introduction to Cell and Tissue Culture", Plenum Press, New York (1998) can also be used. Examples of other enzymes that could be used to digest tissue include neutral proteases, serine proteases including, but not limited to, trypsin, chymotrypsin, elastase, collagenase, and thermolysin. In another preferred embodiment, enzymes that digest DNA, such as DNAase, are used to cut the DNA into smaller pieces in order to prevent tissue aggregation by free DNA. Treatment of fetal pancreatic tissue with enzyme results in cell yields of various amounts. Some cells are in single cell suspensions, others are in cell aggregates. Cells not associated with solid tissue matter can be separated from each other or from solid tissue matter or from debris by using a density gradient. Compounds that can be used to create a density gradient include, but are not limited to, serum (i.e. bovine serum albumin or BSA), ovalbumin, nonionic synthetic polymers of sucrose (i.e. Ficoll™), colloidal polyvinylpyrrolidone-coated silica (i.e. Percoll™), polyvinylpyrrolidone or PVP, and methylcellulose. In a preferred embodiment, density gradients that are capable of neutralizing the enzymes used to digest pancreatic tissues are used. One example of such a density gradient is BSA. The amount of BSA used is about 50% volume-to-volume ratio, more preferably about 25%, more preferably about 10%, and even more preferably about 5%. The amount of debris that needs to be removed depends on several factors, such as the extent of digestion or mechanical shear forces applied to the pancreatic tissue. In some cases, one density gradient is enough to remove debris (e.g. mesenchymal tissue, fatty particles, or broken cell membranes). In other cases, more than one application of a density gradient will be needed. The desired product is a population of relatively pure pancreatic cell aggregates.

Pancreatic cells are then resuspended in a basal cell-sustaining media. A variety of basal cell-sustaining media is available for use. Examples include, but are not limited to, Ham's F12 medium, RPMI-1640, and CMRL-1066. For more optimal conditions to promote pancreatic progenitor cell survival and growth, a variety of nutrients may be added to supplement the basal media. Examples include, but are not limited to, insulin, transferrin, epidermal growth factor, ethanolamine, phosphoethanolamine, selenium, triiodothyronine, progesterone, hydrocortisone, forskolin, heregulin, aprotinin, bovine pituitary extract, and gentamycin. In a preferred embodiment, the following amounts of nutrients are used to promote pancreatic progenitor cell survival and growth: at least about 1 μg/ml insulin and not more than about 100 μg/ml insulin, more preferably about 10 μg/ml insulin; at least about 1 μg/ml transferrin and not more than about 100 μg/ml transferrin, more preferably about 10 μg/ml transferrin; at least about 1 ng/ml epidermal growth factor and not more than about 100 ng/ml epidermal growth factor, more preferably about 5 ng/ml epidermal growth factor; at least about 1×10^{-8} M ethanolamine and not more than about 1×10^{-2} M ethanolamine, more preferably about 1×10^{-6} M ethanolamine; at least about 1×10^{-9} M phosphoethanolamine and not more than about 1×10^{-1} M phosphoethanolamine, more preferably about 1×10^{-6} M phosphoethanolamine; at least about 1×10^{-12} M selenium and not more than about 1×10^{-1} M selenium, more preferably about 1×10^{-8} M selenium; at least about 1×10^{-15} M triiodothyronine and not more than about 1×10^{-1} M triiodothyronine, more preferably about 1×10^{-12} M triiodothyronine; at least about 1×10^{-12} M progesterone and not more than about 1×10^{-1} M progesterone, more preferably about 1×10^{-9} M progesterone; at least about 1×10^{-15} M hydrocortisone and not more than about 1×10^{-1} M hydrocortisone, more preferably about 1×10^{-9} M hydrocortisone; at least about 0.001 μM forskolin and not more than about 50 μM forskolin, more preferably about 1 μM forskolin; at least about 0.1 nM heregulin and not more than about 100 nM heregulin, more preferably about 10 nM heregulin,; at least about 1 μg/ml aprotinin and not more than about 100 μg/ml aprotinin, more preferably about 25 μg/ml aprotinin; at least about 1 μg/ml bovine pituitary extract and not more than about 500 μg/ml bovine pituitary extract, more preferably about 75 μg/ml bovine pituitary extract; at least about 1 μg/ml gentamycin and not more than about 1 mg/ml gentamycin, more preferably about 100 μg/ml gentamycin. The pancreatic progenitor cells may be grown on different substrates, depending on the type of physical orientation of the cells desired. Non-limiting examples of substrates that may be used include fibronectin, laminin, collagen, polylysine, nitrocellulose, nylon, and polytetrafluoroethylene. In one embodiment, pancreatic progenitor cells are grown on fibronectin-coated tissue culture plates in the preferred nutrient media described above. Pancreatic progenitor cells form cell aggregates when cultured in the preferred nutrient media in fibronectin-coated plates. Further, this culturing combination allows for separation of undesired mesenchymal cells and pancreatic progenitor aggregates. In a preferred embodiment, purification of pancreatic cell aggregates is readily accomplished by culturing the pancreatic progenitor cells in preferred media using CMRL 1066 as a basal media in a fibronectin plate. Pancreatic progenitor cells form large, round clusters of cells

that are non-adherent while other cell types (i.e. mesenchymal cells) adhere to the fibronectin coating. The clusters of pancreatic progenitor cells may then be collected and transferred to another tissue culture container for subculturing and proliferation. When proliferation of more pancreatic progenitor cell clusters is desired, the tissue culture container is coated with fibronectin and the pancreatic progenitor cells are cultured in the preferred media disclosed herein using CMRL 1066 as a basal media. In another embodiment, pancreatic progenitor cells are grown in the preferred nutrient media using F12/DMEM as a basal media in collagen-coated tissue culture containers. Pancreatic progenitor cells form monolayers in this embodiment.

The frequency of feeding pancreatic progenitor cells may be once a day or every other day. In one embodiment, pancreatic progenitor cells may be fed by replacing the entirety of the old nutrient media with new nutrient media. In another embodiment, pancreatic progenitor cells may be fed with conditioned media in which these cells were grown. Subculturing pancreatic progenitor cells to obtain a greater number of cells is accomplished by taking pancreatic progenitor cells in cluster form (grown on fibronectin) or in monolayer form (grown on collagen) and dividing the plurality of cells into multiple tissue culture containers. Nutrient media is then added to each of the tissue culture containers to achieve a lower concentration of pancreatic progenitor cells than in the original tissue culture container. The nutrient media that is added is dependent on the type of pancreatic progenitor cell arrangement desired. When monolayer arrangement is desired, then F12/DMEM is used a basal media in the preferred nutrient media disclosed herein coupled with collagen coating in the tissue culture containers. When pancreatic cell clusters are desired, CMRL 1066 is used a basal media in the preferred nutrient media disclosed herein coupled with fibronectin coating in the tissue culture containers. Because the claimed pancreatic progenitor cells are unique to this invention and will secrete factors specific to these cells, the conditioned media derived from the pancreatic progenitor cells are also unique. In this invention, pancreatic progenitor cells form aggregates when grown in the preferred nutrient media, defined above, in fibronectin tissue culture plates. When the substrate is collagen-coated tissue culture plates, pancreatic progenitor cells form an attached stromal monolayer. Addition of conditioned media promotes greater vitality in the pancreatic progenitor cells. A preferred amount of conditioned media is at least about 1% to at least about 25% of total media volume. An even more preferred amount of conditioned media is about 15% of total media volume. A frequency of feeding that is preferable for promoting the survival and growth of pancreatic progenitor cells is once a week, even more preferable is twice a week, and most preferably every other day. The pancreatic progenitor cells of this invention can be passaged multiple times while retaining dividing capability and without inducing differentiation of these pancreatic progenitor cells into terminally differentiated acinar, islet, or ductal cells.

Characterization of Pancreatic Progenitor Cells

The population of pancreatic progenitor cells of this invention isolated in the manner disclosed herein have several defining characteristics. First, the pancreatic progenitor cells are at a stage that can be described as "pre-determined pancreatic". Of the gut-specific progenitor cells, some are pre-determined to become pancreatic cells. It is at this stage of development that the population of pancreatic progenitor cells claimed herein resides (FIG. 8). The pancreatic progenitor cells of this invention have the capacity to become either exocrine or endocrine cells. Endocrine and exocrine cells, as used herein, are defined by their secretions. Endocrine cells, such as α-islet cells and β-islet cells secrete glucagon and insulin, respectively. Exocrine cells, such as acinar cells, secrete a variety of pancreatic digestive juices such as trypsinogen, α-amylase, and lipases.

Identification of pancreatic progenitor cells may be accomplished by morphology or specific markers or a combination of both techniques. As disclosed herein, pancreatic progenitor cells can be rounded and cyst-like in appearance or elongated in a monolayer formation depending on the culture conditions in which the pancreatic progenitor cells are grown. Identification of differentiated pancreatic progenitor cells may also be accomplished by morphology. Morphology of islet cells is an ovoid shape, about 75 μm to 175 μm in size (long axis). Islet cells tend to be located more towards the tail end of a pancreas (away from the duodenal cavity). Markers that can be used to detect islet cells include but are not limited to glucagon for islet-α cells, insulin for islet-β cells, somatostatin for islet-δ cells, and pancreatic polypeptide for islet-PP cells. Markers that can be used to detect ductal cells include, but are not limited to, cytokeratins (CK) 7, CK 8, CK 18, CK 19, mucin MUC1, carbonic anhydrase II, and carbohydrate antigen 19.9 (sialyl-Lewis-a). Morphology of ductal cells is small, round, approximately 10 μm across the cell, appears to be a tightly packed, cuboidal epithelium. Morphology of acinar cells include a larger size than ductal cells, shape, and zymogen granules present within acinar cells. Markers that can be used to identify acinar cells include but are not limited to carboxypeptidase A and amylase.

Ki67 or PCNA are markers that can be used to determine proliferation of pancreatic progenitor cells. Pre-determined pancreatic progenitor cells are still capable of dividing whereas terminally differentiated exocrine or endocrine cells are essentially non-dividing. Staining with Ki67 or PCNA can determine proliferative state of a pancreatic cell under analysis.

Pancreatic progenitor cells of this invention are maintained at their pre-existing pre-differentiation state in serum-free media. Basal cell-sustaining media or the preferred nutrient media disclosed herein or conditioned media may be used to culture the pancreatic progenitor cells in vitro. Different types of substrate on tissue culture plates can be used to obtain either aggregates or monolayers of pancreatic progenitor cells. The use of fibronectin in conjunction with the preferred nutrient media disclosed herein results in aggregates of pancreatic progenitor cells whereas the use of collagen on tissue culture plates results in monolayers of pancreatic progenitor cells.

Pancreatic progenitor cells of this invention have the capacity to be passaged multiple times in the preferred serum-free nutrient media disclosed herein. Multipotency is retained during each passage and at any point after each passage, pancreatic progenitor cells of this invention can differentiate into functional exocrine or endocrine cells. In addition, at any point after each passage, pancreatic progenitor cells may be used as an immunogen, for cell therapy, for bioassays, to establish a human pancreatic model, or for drug discovery and/or development as disclosed herein.

Another characteristic of the pancreatic progenitor cells of this invention is the capacity to differentiate into exocrine or endocrine cells upon transplantation under kidney capsule of a recipient mammal. Prior to transplantation, pancreatic progenitor cells do not make digestive enzymes, such as amylase or lipase, and will not stain positive for digestive enzymes. As disclosed herein, pancreatic progenitor cells

can be grown either in pancreatic progenitor cell aggregates or in monolayers and then combined with mesenchymal tissue and placed under a kidney capsule of a recipient mammal. Preferably, human pancreatic progenitor cell aggregates are combined with rat seminal vesicle mesenchymal tissue and placed under the kidney capsule of a recipient mammal. A portion of the transplant may be removed for analysis using the markers, morphology, or a combination thereof to identify the pancreatic cells.

Antibodies, either monoclonal or polyclonal, which can be used to identify this population of pancreatic progenitor cells include, but are not limited to, anti-cytokeratin 19, anti-carcinoembryonic antigen (CEA), anti-carbonic anhydrase II, anti-cystic fibrosis transmembrane conductance regulator (CFTR).

Uses of Pancreatic Progenitor Cells

Uses as an Immunogen

A use for pancreatic progenitor cells is as an immunogen. As disclosed in this invention, the unique serum-free culturing conditions allow the cell surfaces of the pancreatic progenitor cells to remain free of serum proteins or serum biomolecules that may bind to the surface. A potential problem of antigenic sites that may be "masked" with binding by serum biomolecules is avoided by using the disclosed serum-free isolation and culturing techniques. Accordingly, a panel of antibodies may be generated to newly available antigens that were "masked" when using culture conditions containing serum.

Pancreatic progenitor cells isolated and cultured with the methods disclosed herein can be used as an immunogen that is administered to a heterologous recipient. Administration of pancreatic progenitor cells as an immunogen can be accomplished by several methods. Methods of administrating pancreatic progenitor cells as immunogens to a heterologous recipient include but are not limited to: immunization, administration to a membrane by direct contact such as swabbing or scratch apparatus, administration to mucous membrane by aerosol, and oral administration. As is well-known in the art, immunization can be either passive or active immunization. Methods of immunization can occur via different routes which include but are not limited to intraperitoneal injection, intradermal injection, local injection. Subjects of immunization may include mammals such as mice. The route and schedule of immunization are generally in keeping with established and conventional techniques for antibody stimulation and production. While mice are employed in this embodiment, any mammalian subject including humans or antibody producing cells therefrom can be manipulated according to the processes of this invention to serve as the basis for production of mammalian hybridoma cell lines. Typically, mice are inoculated intraperitoneally or in alternate regions (i.e. footpad, tail base, etc.) with an immunogenic amount of the pancreatic progenitor cells and then boosted with similar amounts of the immunogen. In an alternative, cells grown on non-biological membrane matrix, are surgically implanted intraperitoneally into the host mammal. Lymphoid cells, preferably spleen lymphoid cells from the mice, are collected a few days after the final boost and a cell suspension is prepared therefrom for use in the fusion.

Hybridomas are prepared from the lymphocytes and immortalized myeloma cells using the general somatic cell hybridization technique of Kohler, B. and Milstein, C. *Nature* 256:495–497 (1975) as modified by Buck, D. W., et al., In Vitro, 18:377–381 (1982). Available myeloma lines, including but not limited to X63-Ag8.653 and those from the Salk Institute, Cell Distribution Center, San Diego, Calif., USA, may be used in the hybridization. The technique involves fusing the myeloma cells and lymphoid cells using a fusogen such as polyethylene glycol, or by electrical means well known to those skilled in the art. After the fusion, the cells are separated from the fusion medium and grown in a selective growth medium, such as HAT medium, to eliminate unhybridized parent cells. Any of the media described herein can be used for culturing hybridomas that secrete monoclonal antibodies. As another alternative to the cell fusion technique, EBV immortalized B cells are used to produce the monoclonal antibodies of the subject invention. The hybridomas are expanded and subcloned, if desired, and supernatants are assayed for anti-immunogen activity by conventional immunoassay procedures (e.g., radioimmunoassay, enzyme immunoassay, or fluorescence immunoassay).

Hybridomas that produce such antibodies may be grown in vitro or in vivo using known procedures. The monoclonal antibodies may be isolated from the culture media or body fluids, by conventional immunoglobulin purification procedures such as ammonium sulfate precipitation, gel electrophoresis, dialysis, chromatography, and ultrafiltration, if desired. Undesired activity if present, can be removed, for example, by running the preparation over adsorbents made of the immunogen attached to a solid phase and eluting or releasing the desired antibodies off the immunogen.

In this manner, a panel of novel antibodies to cell surface antigen specific to a stage of pancreatic progenitor cells can be generated using the pancreatic progenitor cells of this invention. Once monoclonal antibodies to cell surface antigens on pancreatic progenitor cells are made by the method disclosed herein, the antibodies can be used to for several uses. The antibodies may be sequenced and cloned for purposes of generating recombinant antibodies or humanized antibodies. Other uses of pancreatic progenitor cell-specific antibodies include but are not limited to biological testing or purification (i.e. isolating pancreatic progenitor cells by methods such as flow cytometry and panning), therapeutic uses (i.e. promoting or arresting cell growth by binding of antibody to target cell or promoting or arresting growth of a cell mass by binding of antibody to target cell), clinical diagnosis, and biological markers (i.e. identification of other pancreatic or non-pancreatic cells).

Another use as an immunogen is to modulate overall immune response in a heterologous recipient. As is well-documented in the art, foreign substances such as cells or organs introduced into a heterologous recipient may induce a variety of immune responses. The immune responses can be in the form of rejection (e.g. in organ transplantation), T cell activation (e.g. cross-priming), anergy, or tolerance. The overall immune response can be systemic or localized. In the case where a localized immune response is desired, for example in the gut region, an immunogen such as pancreatic progenitor cells is introduced into the gut region in an effective amount. Effective amount can be determined in a stepwise fashion in which increasing amounts of pancreatic progenitor cells are introduced into a heterologous recipient and the subsequent immune response is monitored. Overall immune response (e.g. antibody production, cytokine production, T cell proliferation, anergy, tolerance, etc.) may be monitored by a number of methods including but not limited to ELISA, proliferation assays, flow cytometry with cell surface markers, and immunohistochemistry.

Use of Pancreatic Progenitor Cells for Drug Discovery

Another use of pancreatic progenitor cells is related to drug discovery. Since the pre-differentiated multipotent pan-

creatic progenitor cell population has not been isolated and cultured in the disclosed manner, the pancreatic progenitor cell population may secrete proteins that have not been heretofore discovered or characterized. Previous culturing techniques using serum may inhibit the secretion of proteins. Alternatively, proteins may change in function, conformation, or activity as they are being secreted and interacting with serum biomolecules. Proteins secreted by pancreatic progenitor cells have minimal interference from serum biomolecules and thus, may be more physiologically and topologically accurate. Therefore, proteins secreted by pancreatic progenitor cells may be used as targets for drug development. In one embodiment, drugs can be made to target specific proteins on pancreatic progenitor cells in vivo. Binding of the drug may promote differentiation of the pancreatic progenitor cells into specific sub-pancreatic cells, such as islet cells. This approach may be useful when islet cell neogenesis is desired, for example in treatment for diabetes. In another embodiment, drug specific for regulatory proteins of pancreatic progenitor cells may be used to arrest growth of a particular type of cell, for example in cases of cystic fibrosis wherein acinar cells are being replaced by ductal cells. In another embodiment, a drug may be an inhibitor of the growth of stem cells or cancer cells which express fetal antigens. Any of these proteins can be used as targets to develop therapeutic antibody, protein, or small molecule drugs.

Uses of Pancreatic Progenitor Cells for Cell Therapy

In another use, pancreatic progenitor cell lines are used for cell therapy. Transplantation of pancreatic progenitor cells is one such example of cell therapy. In cases where different types of pancreatic cells, such as islet cells or acinar cells, are unable to perform their function of secreting insulin or glucagon respectively, transplantation of pancreatic progenitor cells provides a remedy because the pancreatic progenitor cells of this invention are multipotent and can differentiate into functional exocrine and endocrine cells. To practice this use, pancreatic progenitor cells are isolated and cultured in serum-free, nutrient-defined media using the methods disclosed. Pancreatic progenitor cells are grown on fibronectin-coated tissue culture plates to obtain pancreatic progenitor cell aggregates. Pancreatic progenitor cell aggregates are grown under standard incubation conditions for about half a day to about 7 days, more preferably for about 1 day to about 5 days, and even more preferably about 3 days. Pancreatic cell aggregates can then be administered to a recipient and allowed to differentiate. In an alternative, pancreatic cell aggregates can be used as cellular carriers of gene therapy wherein pancreatic cells are transfected with one or more genes and enclosed in a delivery device and then administered to a recipient. In another embodiment, pancreatic cell aggregates are placed under a kidney capsule and allowed to differentiate into acinar, ductal, or islet cells. In another embodiment, pancreatic cell aggregates are used in a device which contains cells and limits access from other cells (i.e. Theracyte®) to limit immune system responses.

Uses of Pancreatic Progenitor Cells to Make Human Tissue Models

Another use for pancreatic progenitor cells is to create human tissue models in non-human mammals. Pancreatic progenitor cell aggregates are placed on top of mesenchymal tissue to form grafting recombinants. To form grafting recombinants, about 1 to 15 pancreatic cell spheres, more preferably about 5 to 8 pancreatic cell spheres, are placed on top of mesenchymal tissue. The mesenchymal tissue may be either pancreatic or non-pancreatic tissue and may be derived from a different species from which pancreatic progenitor cells are isolated. In a working example, human pancreatic progenitor cells are placed on top of rat mesenchymal seminal vesicle tissue to form a graft recombinant. A skilled artisan may determine the optimal combination in a stepwise fashion, by first isolating human pancreatic progenitor cells using the methods disclosed herein and then combining with mesenchymal tissue from different organs. In some embodiments, a different species, e.g. rat, is used as a source for mesenchymal tissue in combination with human pancreatic progenitor cells. The use of heterologous species allows human-specific markers to be used to determine the identity of differentiated pancreatic cells. The likelihood of false positives is reduced if rat mesenchymal tissue is used. Likewise, the use of seminal vesicle mesenchymal tissue over pancreatic mesenchymal tissue reduces the likelihood of false positives in identifying differentiated pancreatic cells. In a preferred embodiment, about 1 to 12 pancreatic progenitor cell spheres, even more preferably about 5 to 8 pancreatic progenitor cell spheres, are placed on top of rat seminal vesicle mesenchymal tissue. Preferably, about 1×10^4 to about 5×10^6 mesenchymal cells are used. Even more preferably, about 2×10^5 to about 5×10^5 mesenchymal cells are used. A graft recombinant comprising pancreatic progenitor cell spheres placed on mesenchymal tissue is then placed under the kidney capsule, in the fat pad, subcutaneously, or in a device which contains the pancreatic progenitor cells but limits access of other cells to the pancreatic progenitor cells (i.e. Theracyte®) in the recipient mammal. Possible recipient mammals include but are not limited to mice and rats. Typically in graft situations, donor tissue is vulnerable to attack by the recipient's immune system. To alleviate graft rejection, several techniques may be used. One method is to irradiate the recipient with a sub-lethal dose of radiation to destroy immune cells that may attack the graft. Another method is to give the recipient cyclosporin or other T cell immunosuppressive drugs. With the use of mice as recipient mammals, a wider variety of methods are possible for alleviating graft rejection. One such method is the use of an immunodeficient mouse (nude or severe combined immunodeficiency or SCID). In a working example, human pancreatic progenitor cell spheres are placed on rat seminal vesicle mesenchymal tissue and placed under the kidney capsule of an immunodeficient mouse. The graft recombinant remains in the recipient for about 1 to about 52 weeks, preferably about 5 to about 40 weeks, and even more preferably about 6 to about 8 weeks before the grafts are harvested and analyzed for pancreatic progenitor cell differentiation. In some cases, a small portion of the graft is needed for analysis. Markers specific for the islet cells (i.e. insulin, glucagon, etc.), ductal cells (i.e. CK 19, etc.), and acinar cells (i.e. amylase, etc.) is utilized in an immunohistochemical analysis. Another set of markers for exocrine and endocrine functions, such as markers specific for insulin or glucagon, may also be used to analyze the efficacy of the transplantation. These markers can be used separately or in combination with each other. In addition, a combination of one or more of these markers may be used in combination with cell morphology to determine the efficacy of the transplantation.

In one embodiment, human pancreatic model can be generated in a SCID (severe combined immunodeficiency) mouse. This human pancreatic model can be made by utilizing the human pancreatic progenitor cells isolated and cultured with methods disclosed herein and using the human pancreatic progenitor cells to make graft recombinants. Graft recombinants are then placed under the kidney capsule of mice. After about 1 to 10 weeks, preferably about 6 to 8

weeks after implantation under the kidney capsule, the graft or portion thereof is harvested and analyzed by immunohistochemistry. Markers specific to exocrine or endocrine function, such as insulin or glucagon are used to analyze the efficacy of the tissue model system. Alternatively, markers specific for pancreatic tissue such as islet cells (i.e. PDX-1), acinar cells (i.e. amylase), ductal cells (i.e. CK 19) are used. Yet another way to assess the results of pancreatic progenitor cell differentiation is by morphology. Pancreatic progenitor cells have the appearance of being small and round, about 10 μm across the cell, and in a highly compacted columnar epithelium form. Acinar cells have the appearance of large clusters forming acini. Ductal cells have the appearance of small, round, about 40 μm across the cell, and a compacted, cuboidal columnar epithelium. Islet cells have the appearance of epithelial islands surrounded by acinar exocrine units. Further, morphology is combined with functional markers for insulin and glucagon and cell surface markers for specific cells for a more complete assessment. The recombinant tissues thus represent a fully human mini-pancreas in a mouse. These human pancreatic tissue models can be used to assess efficacy and toxicity of drug candidates being developed to treat type I and type II diabetes, pancreatitis, pancreatic cancer, and for other pancreatic insufficiencies. They can also be used to screen any drug for pancreatic toxicity. In a further use, the recipient animal would undergo surgical or chemical (i.e. streptazoticin) pancreatic or islet-β cell ablation so the insulin being produced is coming from the graft.

Uses of Pancreatic Progenitor Cells in Bioassays

The pancreatic progenitor cells disclosed herein can be used in various bioassays. In one use, the pancreatic progenitor cells are used to determine which biological factors are required for differentiation. By using the pancreatic progenitor cells in a stepwise fashion in combination with different biological compounds (such as hormones, specific growth factors, etc.), one or more specific biological compounds can be found to induce differentiation to islet cells. Employing the same stepwise combinations, one or more specific biological compound can be found to induce differentiation to acinar cells and likewise for ductal cells. Other uses in a bioassay for pancreatic progenitor cells are differential display (i.e. mRNA differential display) and protein-protein interactions using secreted proteins from pancreatic progenitor cells. Protein-protein interactions can be determined with techniques such as yeast two-hybrid system. Proteins from pancreatic progenitor cells can be used to identify other unknown proteins or other cell types that interact with pancreatic progenitor cells. These unknown proteins may be one or more of the following: growth factors, hormones, enzymes, transcription factors, translational factors, and tumor suppressors. Bioassays involving pancreatic progenitor cells and the protein-protein interaction these cells form and the effects of protein-protein or even cell-cell contact may be used to determine how surrounding tissue, such as mesenchymal tissue, contributes to pancreatic progenitor cell differentiation.

The following examples provide a detailed description of the isolation, characterization, and use of pancreatic progenitor cells. These examples are not intended to limit the invention in any way.

EXAMPLES

Example 1

Isolation of Pancreatic Progenitor Cells

Fetal pancreas (gestational age 14–22 weeks) was mechanically pulled apart by microdissection under a stereo microscope prior to enzymatic dissociation. Enzyme treatment consisted of placing the partly dissociated tissue in 1 ml F12/DMEM medium containing 5 mg/ml collagenase-dispase, 20 μg/ml soybean trypsin inhibitor and 50 μg/ml DNAase for 15 minutes at 37 degrees Celsius.

Cell aggregates were layered on top of a 5% (by volume) BSA gradient and washed by centrifugation for 6 minutes at 900 rpm. Pelleted cells which were still in aggregate form were resuspended in growth medium consisting of CMRL 1066 nutrient medium containing the following factors:

Insulin	10 μg/ml
Transferrin	10 μg/ml
Epidermal growth factor	5 ng/ml
Ethanolamine	10^{-6} M
Phosphoethanolamine	10^{-6} M
Selenium	2.5×10^{-8} M
Triiodothyronine	10^{-12} M
Progesterone	10^{-9} M
Hydrocortisone	10^{-9} M
Forskolin	1 μM
Heregulin	10 nM
Aprotinin	25 μg/ml
Bovine pituitary extract	75 μg/ml
Gentamycin	100 μg/ml

Resuspended cell aggregates were aliquoted into fibronectin-coated wells (6–12) of a 24-well dish and incubated at 37 degrees Celsius in a humidified 5% CO_2 incubator for 72 hours. After 72 hours, the epithelial cells formed suspended spherical structures (FIG. 1A) and the mesenchymal or stromal cells were attached to the surface of the well. When monolayer formation was desired, the pancreatic aggregates or pancreatic spheres from 6 of the wells were collected with a micropipet and placed on a collagen-coated 60 mm dish using F12/DMEM as basal nutrient media with the nutrients supplements as disclosed. Within 24 hours, the structures attached and the cells from the structure spread out onto the collagen to form an epithelial monolayer (FIG. 1B). These pancreatic progenitor cells could be passaged at least three times (FIG. 2).

Example 2

Use of Pancreatic Progenitor Cells in Transplants

For the purpose of recombinant grafting, the cells were left in the spherical state from the time of original plating or the monolayers were released from the collagen and grown in non-coated flasks where they remained in suspension and re-aggregated into spherical structures.

For the purpose of grafting, the spheres were placed on top of seminal vesicle mesenchyme from e15 rats, usually 5–8 spheres to a mesenchyme aggregate of 2×10^5 to 5×10^5 cells. Each recombinant was placed on agar and incubated overnight at 37 degrees in a 5% CO_2 humidified chamber.

The grafting consisted of placing from 3–6 recombinants under the kidney capsule or fat pad of an immunodeficient mouse (nude or SCID) and left for 6–8 weeks. The grafts were then harvested and processed for immunohistochemistry.

The result of pancreatic tissue recombinant graft transplantation was assessed by morphology. Pancreatic progenitor cells have the appearance of being small and round, about 10 μm across the cell, and in a highly compacted columnar epithelium form. Acinar cells have the appearance of large clusters forming acini (FIG. 3E). Ductal cells have the appearance of small, round, about 40 μm across the cell, and a compacted, cuboidal columnar epithelium (FIG. 3D). Islet cells have the appearance of epithelial islands surrounded by acinar exocrine units (FIG. 3A, 3B, 3C, and FIG. 7).

Example 3

Determining the Identity of Transplanted Pancreatic Progenitor Graft Cells, Differentiation State of Pancreatic Progenitor Cells, and their Function

After the pancreatic spheres have been transplanted under the kidney capsule or fat pad of mice and allowed to remain at that location for 6–8 weeks, the grafts were harvested and analyzed for identity of pancreatic cells by immunohistochemistry and function. The grafts have been shown to express insulin and glucagon (FIGS. **4**, **5**, and **7**). Furthermore, the tissue graft recombinants have shown the formation of ductal structures (FIG. **6**). Therefore, the tissue recombinant grafts yielded functional pancreatic cells that could express insulin and glucagon and form ductal structures.

What is claimed is:

1. A substantially pure population of human pancreatic progenitor cells, wherein said population of pancreatic progenitor cells will differentiate into acinar, ductal, and islet cells.

2. The population of pancreatic progenitor cells according to claim **1**, wherein the pancreatic progenitor cells are isolated and maintained in serum-free media.

3. The population of pancreatic progenitor cells according to claim **1**, wherein said pancreatic progenitor cells are identifiable by the expression of at least one cell marker.

4. The population of pancreatic progenitor cells according to claim **3**, wherein said cell marker is selected from the group consisting of cytokeratin-19, carcinoembryonic antigen, carbonic anhydrase II, and cystic fibrosis transmembrane conductance regulator.

5. The population of pancreatic progenitor cells according to claim **4**, wherein said pancreatic progenitor cells have the morphology of small and round, about 10 μm across the cell, and in a highly compacted columnar epithelial form.

6. The population of pancreatic progenitor cells according to claim **5**, wherein said pancreatic progenitor cells can further differentiate into acinar cells expressing amylase and wherein said acinar cells have the appearance of large clusters forming acini.

7. The population of pancreatic progenitor cells according to claim **5** wherein said pancreatic progenitor cells can further differentiate into ductal cells expressing cytokeratin 19 and wherein said ductal cells have the morphology of small, round, about 40 μm across the cell, and a compacted, cuboidal columnar epithelial form.

8. The population of pancreatic progenitor cells according to claim **5** wherein said pancreatic progenitor cells can further differentiate into islet cells expressing insulin and glucagon and wherein said islet cells have the appearance of epithelial islands surrounded by acinar exocrine units.

9. A method of isolating the substantially pure population of human pancreatic progenitor cells of claim **1**, comprising the steps of:

(a) microdissecting a source of human fetal pancreatic progenitor cells to yield a mixed population of pancreatic cells comprising pancreatic progenitor cells;

(b) placing the mixed population of pancreatic cells in nutrient media under culture conditions sufficient to sustain life of said pancreatic progenitor cells and wherein the nutrient media contains nutrients consisting of: insulin, transferrin, epidermal growth factor, ethanolamine, phosphoethanolamine, selenium, triiodothyronine, progesterone, hydrocortisone, forskolin, heregulin, aprotinin, and bovine pituitary extract;

(c) maintaining suitable culture conditions sufficient to allow pancreatic progenitor cells to form aggregate or monolayer formation; and

(d) subculturing said aggregate or monolayer formation to effect isolation of the substantially pure population of pancreatic progenitor cells.

10. A method of providing a source of an immunogen to a heterologous recipient, comprising introducing a plurality of pancreatic progenitor cells as recited in claim **1** in an amount effective to induce an immune response in said recipient.

11. A method of generating human pancreatic tissue models in an immunodeficient or immunocompromised non-human mammalian recipient, comprising the step of administering human pancreatic progenitor cells of claim **1**, which have been recombined ex vivo with mesenchymal tissue able to effect differentiation of said pancreatic progenitor cells, into said recipient at a location within said recipient able to support growth of said pancreatic progenitor cells.

12. A method of providing a source of pancreatic tissue-specific biological components in a pharmaceutical development of one or more drugs comprising isolating the population of human pancreatic progenitor cells as recited in claim **1**, and using said pancreatic progenitor cells or any cellular part of the cells thereof as targets of one or more drugs under development.

13. A method of providing a source of nucleic acids or proteins in a development of bioassays comprising isolating nucleic acids or proteins from the human pancreatic progenitor cells as recited in claim **1** and using said nucleic acids or proteins as one or more of the principle component in the bioassays.

* * * * *

B

PATENT
Docket No. 415072000200

CERTIFICATE OF MAILING BY "FIRST CLASS MAIL"

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on August 9, 2000.

Kimberly Benjamin

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Penelope E. ROBERTS and Jennie Powell MATHER

Serial No.: 09/546,577

Filing Date: April 10, 2000

For: HUMAN PANCREATIC EPITHELIAL PROGENITOR CELLS AND METHODS OF ISOLATION AND USE THEREOF

Examiner: Not Yet Assigned

Group Art Unit: 1643

INFORMATION DISCLOSURE STATEMENT UNDER 37 C.F.R. § 1.97

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Pursuant to 37 C.F.R. § 1.97 and § 1.98, Applicants submit for consideration in the above-identified application the documents listed on the attached Form PTO-1449. Copies of the documents are also submitted herewith. The Examiner is requested to make these documents of record.

pa-499572

This Information Disclosure Statement is submitted:

☒ Within three months of the application filing date or before mailing of a first Office Action on the merits; accordingly, no fee or separate requirements are required.

☐ After receipt of a first Office Action on the merits but before mailing of a final Office Action or Notice of Allowance.

- ☐ A fee is required.
- ☐ A Certification under 37 C.F.R. § 1.97(e) is provided below; accordingly; no fee is believed to be due.

☐ After mailing of a final Office Action or Notice of Allowance, but before payment of the issue fee. Accordingly, a Petition requesting consideration of the Information Disclosure Statement, an authorization to charge our deposit account, and a Certification under 37 C.F.R. § 1.97(e) are provided herein.

Applicants would appreciate the Examiner initialing and returning the Form PTO-1449, indicating that the information has been considered and made of record herein.

The information contained in this Information Disclosure Statement under 37 C.F.R. § 1.97 is to the best of my knowledge and is not to be construed as a representation that: (i) a complete search has been made; (ii) additional information material to the examination of this application does not exist; (iii) the information, protocols, results and the like reported by third parties are accurate or enabling; or (iv) the above information constitutes prior art to the subject invention.

In the unlikely event that the Patent Office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing

415072000200. However, the Assistant Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: August 8, 2000

Respectfully submitted,

By: _____
Terri Shieh-Newton
Registration No. P-47081

Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, California 94304-1018
Telephone: (650) 813-5649
Facsimile: (650) 494-0792

PTO/SB/08 (2-92)
Sheet 1 of 1

Form PTO-1449	Docket Number 415072000200	Application Number 09/546,577
INFORMATION DISCLOSURE CITATION IN AN APPLICATION	Applicant: Penelope E. ROBERTS and Jennie Powell MATHER	
(Use several sheets if necessary)	Filing Date April 10, 2000	Group Art Unit ~~1643~~ 1632
	Mailing Date August 9, 2000	

U.S. PATENT DOCUMENTS

Examiner Initials	Ref. No.	Date	Document No.	Name	Class	Subclass	Filing Date If Appropriate
JW	1.	11/10/1998	5,834,308	Peck et al.	—	—	
JW	2.	03/30/1999	5,888,705	Rubin et al.	—	—	

FOREIGN PATENT DOCUMENTS

Examiner Initials	Ref. No.	Date	Document No.	Country	Class	Subclass	Translation YES	Translation NO
JW	3.	05/01/1997	WO 97/15310	WIPO	—	—		

OTHER DOCUMENTS *(Including author, title, Date, Pertinent Pages, Etc.)*

Examiner Initials	Ref. No.	Title
JW	4.	Barnes et al., (1980). "Methods for growth of cultured cells in serum-free medium" *Anal. Biochem.* 102:255-270.
	5.	Buck et al., (1982). "Monoclonal antibodies specific for cell culture mycoplasmas" *In Vitro* 18(4):377-381.
	6.	Freshney, R.I., (ed), (1987). Animal Cell Culture pp.vii-xii. (Table of Contents).
	7.	Ganong, William F., (1991). "Regulation of gastrointestinal function" *in* Review of Medical Physiology, fifteenth edition, Appleton and Lange, (eds), Chapter 26, pp. ~~448-447~~.
	8.	Ham et al., (1979). "Media and growth requirements" *Meth. Enzy.* 58:44-93.
	9.	Harlow and Lane, (eds), (1988). Antibodies, A Laboratory Manual pp.iii-ix . (Table of Contents).
	10.	Köhler et al., (1975). "Continuous cultures of fused cells secreting antibody of predefined specificity" *Nature* 256:495-497.
	11.	Mather, Jennie P. and Roberts, Penelope E., (1998). Introduction to Cell and Tissue Culture Plenum Press, New York pp. xi-xiv. (Table of Contents).
	12.	Olumi et al., (1999). "Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium" *Cancer Research* 59:5002-5011.
	13.	Stephan et al., (1999). "Selective cloning of cell surface proteins involved in organ development: Epithelial glycoprotein is involved in normal epithelial differentiation" *Endocrinology* 140:5841-5854.

EXAMINER: Joel Weitzach DATE CONSIDERED: 4/10/01

EXAMINER: Initial if citation considered, whether or not the citation conforms with MPEP 609. Draw a line through the citation if not in conformance and not considered. Include a copy of this form with next communication to applicant.

JDRF C

dedicated to finding a cure

May 27th, 2003

Jan Visser, Ph.D.
ViaCell, Inc.
131 Clarendon Street
Boston, MA 02116

Title of Application: Adult human islet-derived pancreatic stem cells (PSCs) as a substrate for the *ex vivo* manufacture of a cellular product for the treatment of diabetes

Dear Dr. Visser:

On behalf of the Board of Directors of the Juvenile Diabetes Research Foundation International (JDRF), I regret to inform you that JDRF will be unable to support the above-named research project. A summary of the reviewer critiques and recommendations is attached. This decision was based on a number of parameters, which are outlined in this summary statement.

Please note that the review committee did not solicit resubmission of your application to JDRF.

However should you choose to submit a revised version of this application it must be submitted first as a Letter of Intent (LOI). Please be advised that our next LOI deadline is June 30th, 2003. A copy of our updated Industry LOI RFA is attached.

Please contact me if you have any questions.
Sincerely,

Kim Hunter-Schaedle, Ph.D.
Associate Director, Industry Relations
khunter@jdrf.org Tel: 212-479-7537

cc: Robert A. Goldstein, MD, Ph.D., Chief Scientific Officer
Charles Queenan III, Chair of Research
JDRF File

dedicated to finding a cure

JDRF Industry Grants

dedicated to finding a cure

JDRF Industry Grants

Companies intending or considering submission of a grant application to JDRF are recommended to first contact Kim Hunter-Schaedle, Ph.D., Associate Director, Industry Relations (Phone: 212-479-7537; email: khunter@jdrf.org).

Purpose

JDRF Industry Grants are intended to support applications from companies or for-profit entities proposing research programs that are targeted to one of JDRF's priority areas and closely focused on JDRF's mission. Areas of scientific need as outlined in the RFA - Research Emphasis Areas should be addressed. In particular JDRF encourages applications to develop or test, in preclinical models or early-stage clinical trials, novel therapeutic approaches for diagnosis, prevention or treatment of Type 1 diabetes or its complications.

Eligibility

JDRF Industry Grants are intended to support research in biotechnology companies or other for-profit entities, either publicly or privately held. JDRF also encourages applications to this category from companies proposing collaboration with academic researchers.

Companies approved for funding by JDRF will be expected to enter into a contract-based agreement with JDRF regarding anticipated milestones in relation to anticipated funding, intellectual property and royalty issues, etc. These will be negotiated on a case-by-case basis and it is expected that these agreements will be in place before the commencement of funding.

Mechanism of Support

JDRF Industry Grants will be supported to a maximum of $1 million total costs per year for a period of up to 3 years. Applicants are however encouraged to consider requesting in the first instance smaller funding amounts to support short-term studies (e.g. 1 year) that will provide data and a foundation on which to base a request for additional funding, either from JDRF or alternate funding sources.

Note: Indirect costs are not allowable on direct funding to for-profit entities: these are only allowable on academic sub-contracts and cannot exceed 10% of direct costs.

Matching Funds

Applicant companies must demonstrate a fiscal commitment to the proposed project that is equal to or greater than the fiscal amount requested from JDRF.

Industry Grants applications must be preceded by a Letter of Intent (LOI). These are reviewed in 2 cycles annually as follows:

LOI to be received by:	LOI review decision:	Full application due by:	Review decision notification:
6/30/03	mid-July 2003	9/15/03	early December 2003
12/31/03	mid-January 2004	3/15/04	early June 2004

Letter of Intent (LOI) Application
The letter of intent should outline briefly the goal of the proposed study, relevance to JDRF mission, where it fits in the company business plan, estimated cost, timeframe and milestones, budget requested (linked to milestone attainment), other sources of company funding and details of matching funds to be commited to this project, and the biographical information on the principal investigator and co-investigators.
The LOI should be 5-10 pages maximum.
Please submit the signed letter of intent by dates indicated above to:

> Dr. Kim Hunter-Schaedle, Associate Director, Industry Relations, Juvenile Diabetes Research Foundation International, 120 Wall Street, 19th Floor New York, NY 10005-4001 USA

An electronic copy should also be sent to khunter@jdrf.org.

Notification of LOI Decision
Applicants will be notified in the timeframe indicated in the table above as to whether the LOI has been approved and a full application is solicited. Additional guidelines on preparing your application for submission will be provided at this time.

Juvenile Diabetes Research Foundation International

dedicated to finding a cure

JDRF Industry Grant Review Spring 2003
Summary Statement

Applicant: **Jan Visser, Ph.D.**
ViaCell, Inc.
131 Clarendon Street
Boston, MA 02116

Title of Application: Adult human islet-derived pancreatic stem cells (PSCs) as a substrate for the *ex vivo* manufacture of a cellular product for the treatment of diabetes

Note to Applicant: Your application was reviewed according to the policies and procedures of JDRF for Industry Grant Applications which are as follows: I) Scientific review; and II) Lay Review followed by (for applications deemed meritorious following these two tiers of review) III) Business Review; and IV) review by our Board of Directors who are responsible for final decisions. While it is not possible to provide you with details of all of the discussions of these review panels, we are able to provide you with the following summary statement, which is intended to represent constructive criticisms concerning your application. If you have any questions about this critique or about JDRF policy and procedures, please contact a member of the JDRF Research Staff.

Outcome of Review

As a result of the considerations summarized below, the review panel recommended that this application does not merit funding in its current form.

Summary of Scientific and Lay Review

The reviewers noted that this application proposes to generate human beta cells from endogenous pancreatic stem cells and test these in transplant models of diabetic mice and primates. The reviewers felt that overall the application is innovative; that the proposed approach with adult stem cells is unique; and that the proposed studies with tolerization were potentially exciting and in theory may yield positive results.

However there were a number of concerns. ***The reviewers felt that overall the submission of the application is largely premature due to a fundamental lack of supporting preliminary data that is required to support the novel and somewhat controversial hypothesis presented.***

The reviewers noted that the hypothesis presented is novel and somewhat controversial, since it explores a new research area. In the light of this the reviewers felt the onus was on the applicants to ensure that a rigorous approach be taken to these studies. The reviewers felt that neither the preliminary data presented nor the studies outlined were sufficiently rigorous.

The reviewers were concerned that there was no demonstration that nestin expressing cells are indeed stem cells. Published reports from Edlund and others, whose papers presented data to show that nestin is not expressed in beta cell precursors, were not mentioned or cited. It is ridiculous that they do not comment on this paper at all. In view of the fact that the application under review was utilizing nestin positive cells for islet source generation, the reviewers felt it was necessary for the applicants to have addressed the data presented in these papers.

The applicants propose to measure insulin production by the cells but no preliminary data – even a measure of insulin/mg protein – is presented. The amount of insulin that would be produced by these cells is in fact likely to be extremely low, but this issue is not discussed.

Overall the reviewers were concerned about the lack of data and the fact that there is no data that the cells to be the focus of this study either would or could cure diabetic mice. It was not clear that ViaCell would advance beyond the original findings that formed the basis of this application.

Budget: the reviewers felt this was excessive especially in terms of personnel for who support is requested. The reviewers also noted that the applicant company has 2 locations: one being better equipped than the other: and that in part funding is requested to equip the less well equipped site. The reviewers felt this was not necessary and that instead much of the proposed work could be consolidated to one site.

Note: As a result of this recommendation your application did not undergo Business Review.

Abstract of Application

ViaCell Inc proposes to generate human beta cells from pancreatic stem cells. This proposal is based on works performed in the laboratory of Joel Habener in Boston, where pancreatic stem cells were characterized based on their expression of the intermediate filament Nestin. Such nestin positive cells are detected in the pancreas of adult rodents and humans and can be isolated and expanded in vitro. It also seems that such cells have some potential to differentiate into beta cells. An important point is that such stem cells are immunologically privileged. ViaCell has licensed this technology that represents the key point of the proposal.

There are 3 aims in this proposal:

-1- Isolation of pancreatic stem cells and engineering of a clinical scale system for the ex vivo expansion of pancreatic stem cells and their derivates

-2- Transplantation of pancreatic stem cells and their derivates in diabetic mice and monkeys to evaluate their function

-3- Induction of immune tolerance using pancreatic stem cells.

Individual Reviewer Critiques

CRITIQUE – Reviewer 1:

Overall summary of critique:

This proposal is based on works performed in the laboratory of Dr Habener. While the work published by the group of Dr Habener is interesting, it is not yet fully accepted by the scientific community. For example, published works performed by the group of Helena Edlund in Sweden or Luc Bouwens in Belgium strongly suggest that nestin-positive cells present in the pancreas are not pancreatic stem cells. Significantly, such points are not discussed in the present proposal. In addition, while it is clear that insulin mRNA can be detected by PCR in cultures of nestin-positive cells, the amount of insulin per nestin-positive cells is not discussed here. There is a possibility that nestin-positive cells only contain traces of insulin. Finally, no evidence is presented suggesting that nestin-positive cells or their derivates could cure diabetic mice. Such preliminary data is crucial.

Rationale: Here the objective is to use pancreatic stem cells (located in the pancreas) as a source of stem cells to generate mature and functional beta cells. Such pancreatic stem cells are the ones that physiologically differentiate into beta cells during the whole life. They do thus have by definition the capacity to differentiate into beta cells.

Research design and Methods: The strategy proposed is based on a protocol recently designed in the laboratory of Dr Habener.

The first step is to purify pancreatic stem cells located within human islets. Human islets prepared in Edmonton will be plated and using a protocol designed by Dr Habener, nestin-positive cells that are the potential stem cells will migrate from the islets. Such cells will next be expanded. The feasibility of such step is high.
The next step is to isolate "enriched populations of stage specific cells by FACS. Different positive and negative markers will be employed. The rationale for the choice of such positive and negative markers is unclear and references are not given.
The next step is to scale up the system. ViaCell Inc should be able to perform this industrial step.
The next step is to differentiate such pancreatic stem cells into "islet-like clusters". The applicants indicate that up to now islet like cluster formation is consistent but highly variable. They want here to standardize islet-like cluster formation. It is also proposed to measure here insulin content. Preliminary data showing insulin/mg protein of islet like clusters is missing key data.
The final step is to transplant adult derived pancreatic stem cells and their derivates into models of diabetes. Different routes of injection will be compared (intravenous tail, hepatic portal vein, intrasplenic, subrenal). The rationale for each site is well described. Both diabetic mice and diabetic non-human primates will be transplanted. The model in mice is currently functional. The model in non-human primate is correct.

Preliminary data: The first group of preliminary data is derived from the laboratory of Dr Habener, generated by Dr Abraham when she was post doc in Habener's lab. She confirmed in ViaCell the data obtained in Habener's lab. In brief, nestin-expressing cells can be enriched from human islets and RNA coding for insulin can be amplified from such cells. *Defining the amount of insulin per cell would have strongly strengthened the preliminary data.*
The nestin-expressing cells were next transplanted to non-immunosuppressed mice. The cells grew and were not rejected. Such 2 points are important. It seems that islands of endocrine tissue developed, but the picture that is presented is not fully convincing. *Insulin content per graft, in situ hybridization using an insulin probe, demonstration that human insulin or human C-peptide is found in the blood of the mice would have strongly strengthened the preliminary data.*
Data related to FACS analysis are presented indicating the feasibility of this step.
Finally data indicating that diabetic mice and non-human primates can be generated are presented.
In conclusion, preliminary data are clear. *Crucially, what is missing is a demonstration that nestin-expressing cells are indeed stem cells.*

Research milestones: The milestones are clear and logic. A new time, preliminary data should have been presented related to the milestone "evaluate PSC in STZ induced mice". It is however not clear that the non-primate part can be started year 1.

Resources and Environment: Resources and environment seem perfect to reach the objectives.

Personnel: 22 people are involved in this project. Dr Abraham did a recent post doc in the laboratory of Dr Habener. Two scientists have an excellent background in the field of stem cell research.

Contractual collaborative arrangements: Strong collaborations exist with Dr Habener. Collaboration has also been built with Dr D. Greiner (Division of Diabetes, Worcester), to generate models of diabetic mice and for transplantation.

Budget: 22 salaries are requested which is a lot. $300,000 for supplies for year 1 is also very high. Equipment: $237,000 requested. It is not clear why 4 CO2 incubators are needed.

Matching funds: The total budget for 3 years is 11,623,673 million dollars and 3 millions are requested from JDRF.

Assurances: Copies of the institutional review board have not been included.

Outcome benefits of research: If human pancreatic stem cells could be use as a starting source to generate human beta cells, this would be a dream. In addition, such human pancreatic stem cells are not rejected when grafted to immunocompetent mice, suggesting that they could be used without immunosuppression.

Information disclosure and sharing of generated resources: This issue was difficult to judge.

CRITIQUE – Reviewer 2:

Overall summary of the evaluation: This study is based on the assumption that nestin-positive islet derived progenitor cells (PSCs) participate in islet neogenesis. Such cells neither express insulin nor glucagon and they are different from ductal epithelial because they do not express CK19.
The study has got three major aims:
1; To isolate enriched preparations of adult human multipotent PSCs and engineer an ex vivo system for their amplification
2; To evaluate in animal models of diabetes the feasibility of conducting a clinical trial
3; To induce immune tolerance by using MHC negative PSCs in an animal model

The major problem with this study is that there are at least two publications last year (Helena Edlund's group and Hanley in Newcastle), which very thoroughly have investigated this working hypothesis. Both of them concluded from their data, quite in contrast to the studies by Habener et al, that they call for caution in trying to generate beta cells from nestin-positive cells. The applicants apparently prefer not to discuss these quite intriguing data.
One more weakness of the application is that the transplantation experiments are anecdotal in nature and that they have not been published. This also means that the preliminary data on induction of immune tolerance are difficult to judge.

Rationale: The proposal addresses an important issue but for the reasons given above one has to be quite sceptical as regards the scientific basis for the study (negligence of discussing reports not supporting the basis for the whole grant proposal). A proposal on just the propagation of the PSCs and their molecular characteristics would have been more appropriate.

Research Design and Methods: Details of this kind are quite sparse (1 page on how PSCs are prepared and qualitatively scrutinized). The transplantation studies are described more in detail. To some extent the applicants seem to rely on the efforts of the consultants (Dale Greiner; Charles River Laboratories).

Preliminary data: The platform for this proposal is the publication by Habener et al (Diabetes, 2001). One of the collaborators of that study, Dr Elizabeth Abraham, has joined the company since then and the preparation technology now seems to work in the new environment. Some improvement seems to have been implemented re the isolation/preparation of PSCs. FACS analysis has been carried out in order to describe the heterogeneity of the PSC cultures. There are also anecdotal reports on non-immunosuppressed mice successfully transplanted with human PSCs. The xenogeneic grafts were shown, by immunohistochemical means, to contain insulin and glucagon. They also contained PDX-1 positive cells and there were no signs of rejection.

Research milestones: Keeping in mind the doubt of this reviewer and the scientific community in a broader sense to the basic idea of this study it does not make sense to comment on this issue.

Resources and Environment: ViaCell seems to be a well-equipped company but still equipment applied for in the project amounts to §240.000. Why should two laboratories be equipped?

Personnel: The PI is a recognized stem cell biologist. His experience in experimental diabetes research, however, is minimal. In general, his scientific productivity, shown as being the responsible authors of scientific papers, is limited over the last years. Dr Elizabeth Abraham, senior scientist and Co-PI, has been working in the lab of Joe Habener over the last years with the Nestin project. Her qualifications from other areas are difficult to judge and especially so

in the field of diabetes. This is somewhat unsatisfactory, since she is the person supposed to do the job. Dr Dale Greiner, UMASS, has expressed his delight in collaborating in the rodent transplantation experiments.

Contractual Collaborative Arrangements: Dr Habener is the only consultant specified. There is quite a costly contract with him but most certainly it is necessary for the project.

Budget:
The budget requested is difficult to assess. The Co-PI and one technician are the only personnel working on the project full time at present. Four more are to be named. A considerable sum is the cost for the animal care, especially so the monkeys. These costs are not appropriate at present. Contractual costs: the only person named this far is expensive.
No other support is reported.

CRITIQUE – Reviewer 3:
This review is focused on the proposed cell culture aspects, for which extremely limited detail has been provided.

The multiple difficulties that will likely arise while translating mainly qualitative laboratory results to the stringent needs of clinical applications are essentially not addressed.

Rationale: The in vitro production of islets has the potential to cure diabetes. Production based on adult islet-derived PSCs deserves further research and development efforts, particularly if such an industrial group will address the many practical issues that most academics groups neglect.

Preliminary Data: The culture expansion of putative PSCs has been replicated and extended to "large numbers" and for months, but the neither the quantity nor the quality of the generated cells is not at all clear. In most cases of this type, the quality of the cell populations is greatly deteriorated in culture. Parallel hematopoietic stem cell (HSC) work might give some confidence in the capabilities of this company, but again, almost no performance or scale data is given. (The 40- to 150-fold HSC expansion cited in the Business Plan is incredible and must be regarded with caution since even the refereed literature is full of such reports, though no such major expansion has been confirmed by rigorous in vivo assays.)

Research Design and Methods: The proposal to focus on expanding PSCs is a good one. Selection strategies are a valid aspect of such an effort and a core strength of this company. However, the predominant focus on the selection aspect for the process development seems inappropriate. The single paragraph on "Process Engineering Development and Scale Up of Ex vivo Amplification

Systems" makes no mention of further cell culture optimization strategies (e.g. factor additions that might be tested). Based on the mainly qualitative preliminary data, there is little evidence presented that the medium composition has been optimized and so this aspect should have been addressed in the proposal. Furthermore, although selection strategies may be valuable, they will be useless if they cannot be practically scaled-up. How these will be scaled-up is not addressed except that a year and a half effort is cited in the milestones. Overall, I would expect the otherwise neglected aspects of cell culture and selection scale-up to be major engineering challenges.

Research Milestones: These are overly ambitious.

Resources and Environment: Though "all equipment needed for large scale cell cultures" is cited, it is not described.

Personnel: The personnel seem very good, though the cell culture scale-up engineering expertise appears weak. The linkage with Dr. Habener is excellent.

Budget: The budget appears overly modest for the work described.

Outcome Benefits of Research: The benefits could be very great, but it is not at all clear how close this group's current results may be to clinical applications.

CRITIQUE – Reviewer 4:

Overall assessment: This proposal is scientifically exciting and unique in the approach to use adult stem cells that are derived in pancreatic islets. A clear approach towards non-human primate experiments is taken as assessment of feasibility towards human trials.
However, the application is deemed premature. Its drawbacks are mainly the lack of clear and conclusive preliminary data. A better phenotypic characterization of the PCS and ILC at the genetic level (ie markers of stem cells such as oct-4, telomerase), and more detailed analysis of expressed genes/proteins, are needed but are lacking. Furthermore, the potentially important observation that these PCS may evade immune rejection or/and induce tolerance needs to be better substantiated at the molecular level and possibly with in vitro assays (such as T-cell activation, Cr-51 release assays). The observation that these cells continue to proliferate after xenotransplantation into mice needs further clarification as to whether this expansion in cell number is an adaptive response or an uncontrolled proliferation of these cells that are placed under the kidney capsule. On this basis it appears premature to propose trials in non-human primates. A better characterization in the mouse xenotransplantation model would greatly benefit a reapplication of this proposal.

Rationale: The present grant proposes generation of large quantities of transplantable tissue that functionally behaves like pancreatic islets. The primary source of cells are adult human islet-derived nestin positive pancreatic stem cells (NIPS or PCS) for a) in vitro expansion, b) in vitro differentiation of islet like clusters (ILC). These cells have been described and characterized in the laboratory of Joel Habener, who is a consultant in the present proposal. These PCS and ILC are reported to have properties very much like pancreatic beta cells in their secretory capacity of insulin and certain electrophysiological properties after stimulation with glucose and glucagon-like peptide-1. Furthermore the applicants claim that these PCS and ILC evade immune rejection in that they do not express MCH class I and II surface molecules and that they induce mixed chimerism. On this basis the applicants are moving proposing injection/transplantation of PCS or ILC into rodent and non-human primate models of streptozotocin-induced diabetes mellitus to assess the effectiveness of this approach in a cell-based cure for type 1 diabetes mellitus. A strength of this proposal – only indirectly mentioned – is that ILCs may be capable of mimicking whole islets with all the islet hormones being present in different cells of the ILC. This approach differs from other approaches that aim to generate pancreatic beta cells only.

Research Design: The research design is straightforward. The investigators propose to grow PCS and ILC, transplant them into diabetic mice and non-human primates with or without immune suppression and follow metabolic control. The methods to isolate, culture and differentiate PCS is well presented and published by the co-PI. The main drawback to the research design is the lack of enough detailed preliminary data to support the advancement of the work into the time and cost-intensive non-human primate model.

Preliminary Data: The proposal builds on the finding that nestin-expressing cells are present in pancreatic islets of rodents and humans. These nestin expressing cells can be isolated from islets by culture methods or by fluorescent activated cell sorting. The former has the potential to damage DNA and is therefore not proposed to be a viable approach in the beginning of the proposal. However, later in the proposal it is then again introduced as a method for isolating the NIPs. These NIPs can be passaged for several months and under certain culture conditions will form small cell clusters that express hepatic and pancreatic markers, secrete insulin upon glucose and glucagons-like peptide 1 stimulation. Data is presented on xenograft experiments of human PCS under the kidney capsules of C57Bl/6 mice, where expression of islet hormones and the pancreatic transcription factor PDX-1 is detectable. Although hepatic markers are mentioned in the initial report of PCS that differentiate into endocrine cells, no hepatic markers are mentioned in the in vivo xenotranplantation experiment in mice. The applicants point out that no rejection of the xenografts was noted. Furthermore, it is mentioned that the PCS lack MHC class I and II molecules. Immunohistochemistry data show that nestin-positive PCS lack MHC I and MHC II when examined in rat islets. However, no clear positive control for MHC I and II

staining is provided . Furthermore, no data on MHC expression (RNA and protein) is provided at from NIPs and ILCs taken and grown in culture and there is no staining for MHC I and II of NIPs derived tissue under the kidney capsule of transplanted mice. The applicants propose mechanisms how the PCS evade immune-rejection but fail to provide convincing data for their contentions.
Data of streptozotocin induction of diabetes in NOD/SCID mice and successful treatment of a subset with insulin pellets are shown. Data of successful induction of diabetes mellitus with streptozotocin in Cynomglus is presented.

Research Milestones: A schematic on the proposed time-course of the studies is provided on page 5 of the application. There is no other assessment of milestones specifically mentioned in the proposal. However, it is assumed that metabolic correction of diabetic animals after transplantation of PSC or ILC will be important milestones in the studies. The time course proposed appears rather optimistic. It is doubtful that all the proposed work – in particular the time-intensive non-human primate work - can be conducted in the three years that are applied for. Because of concerns noted further below, it is doubtful that the research milestones can be achieved in the presented time frame. It is suggested that a more detailed assessment of the growth characteristics of PCS after transplantation, immune-phenotype of PCS and phenotype assessment (liver phenotype, islet phenotyp) be conducted before the non-human primate xenotransplantation work be conducted.

Resource and Environment: ViaCell has two campuses. The campus in Worcester, MA appears to be better equipped than the one in Cambridge, MA. Overall, ViaCell appears to be well equipped with the infrastructure to conduct the work proposed.

Personnel: The PI and the Co-PI are well equipped to lead and conduct the proposed work. Dr. Visser has devoted his career to stem cell biology and has a tremendous experience in this field. Dr. Abraham has worked in Dr. Habener's laboratory and received first-hand experience with NIPS and ILC under Dr. Habener's guidance before moving to Viacell when the NIPS project was transferred to industry. A number of other individuals are involved in the development of the project including one full time technician, one 50% scientist and still to be named scientists (2), Sr. research associate (1, 25%) and technicians (2).
A program manager is included in the staff with a large percentage of effort. The role of the program manager is not clearly delineated in the proposal and it is unclear why a relatively large amount of salary is being dedicated to this function. The key personnel mentioned on page 3 of the application is adequate to perform the proposed work.

Contractual and Collaborative Arrangements: Although the applicants mention that human pancreatic islets will be supplied by the group in Edmonton, Alberta, Canada, there is no documentation provided that the group in Edmonton

agrees to supply the material needed for the successful implementation of the proposed projects.
Mouse work will be conducted at the University of Massachusetts in Worcester with a collaborative agreement with Dr. Greiner. The animal facility is located close to the ViaCell campus in Worcester and is experienced in the conduct of the proposed work. No approval is provided for the conduct of mouse experimentation with this proposal.
Non-human primate (Cynomolgus Monkey) work is proposed as a contract to Charles River Laboratories. A part of the present proposal seeks funding for application for approval for conducting studies in non-human primates. It is assumed that if funded, the application for non-human primate studies will be initiated.
Dr. J. Habener is a consultant for all aspects of the studies proposed in the present application. Dr. Habener is a preeminent researcher in the diabetes field and his experience and expertise are a tremendous asset to the present project.

Budget: There are some concerns:
The proposal requests investment for equipment of the Cambridge location (cryomicrotome, fluorescence microscope, film developer, imaging scope system, microplate reader, gamma counter and spectrophotometer). The prices mentioned for these machines are at the higher end of the ranges that are currently available. Rather than purchasing this new equipment, the applicants may consider to conduct most of the work proposed at the Worcester campus, which is closer to the mouse and non-human primate facilities. A rationale to specifically equip the Cambridge campus is not given.
A more detailed rationale for involving a program manager with a relatively high percentage contribution needs to be provided.
It may be unrealistic to advance into non-human primate models before addressing in the mosue model important aspects of growth behavior and immune phenotype of PCS and ILCs. Therefore, proposing the non-human primate aspect and its budget is premature.

Matching Funds: Viacell proposes to invest twice the absolute dollar amount towards the project as is being sought for from JDRF. There are no competing grant applications mentioned. In the event of commercially profitable outcome of the proposed project, ViaCell pledges to remunerate JDRF according to "industry standards".

Assurances: Precautions towards hazards for potential human application of the present proposal are not clearly addressed in the present proposal. Below are a few examples deemed important by the reviewer:

Special note on immune tolerance: There are no studies proposed to assess the immune rejection/tolerance of the transplanted pancreatic stem cells or ILC. The reviewer finds that rejection or tolerance should be positively documented by

experimental means in the present proposal for successful development of the proposed procedure towards a therapy.
Growth behavior of transplanted cells/ILC: Which precautions and tests have been taken and will be taken to assure that the NIPS and ILC's do not transform into malignant growth behaviour?
Infection control: It is assumed that information from organ donors regarding infections agents will be provided when procuring the islets used in the proposal. Further testing is not proposed in the present application.

Outcome/Benefits of Research: The outcomes of the studies may lead to a conclusion whether the pancreatic stem cells indeed can be used as a source for transplantable human tissue in diabetics. The study design directly addresses the question about what may happen in the xenotransplantation model. The concerns are that insufficient studies are proposed to address the scientific concenrns mentioned elsewhere in this evaluation.

Information Disclosure and Sharing of Generated Resources: On page 61 of the proposal ViaCell assures that full access to data and findings will be provided. Scientific findings will be reported in peer-reviewed journals and via presentations at scientific meetings. ViaCell pledges to share reagents with prevailing industry and academic standards. The PI on the project has published in peer-review journals from his position at ViaCell in the past, and it is anticipated that he may continue to do so in the future.

D

MASSACHUSETTS GENERAL HOSPITAL

HARVARD MEDICAL SCHOOL

Laboratory of Molecular Endocrinology
55 Fruit Street, WEL 320
Boston, MA 02114-2696

E-mail: jhabener@partners.org
Tel: 617.726.5190. Fax: 617.726.6954

Joel F. Habener, M.D.
Professor of Medicine
Harvard Medical School
Chief, Laboratory of Molecular Endocrinology
Massachusetts General Hospital

Thursday, January 20, 2000

Gordon C. Weir, M.D.
Editor, Diabetes
Joslin Diabetes Center
One Joslin Place
Boston, MA 02215

Dear Dr. Weir,

My colleagues and I submit the enclosed manuscript entitled "The Neural Stem-Cell Protein Nestin Expressed in a Distinct Cell Population Within Pancreatic Islets" for your consideration for publication as a Rapid Publication in *Diabetes*. We describe the identification within islets of stem/progenitor cells that express the neural stem-cell specific marker nestin, and that proliferate *in vitro* for nine months. We also have preliminary evidence that glucagon-like peptide agonists stimulate the proliferation of these islet stem/progenitor cells.

Sincerely yours,

Joel F. Habener

Joel F. Habener, M.D.

JFH/ral

diabetes

JOURNAL OF THE AMERICAN DIABETES ASSOCIATION

Joslin Diabetes Center • One Joslin Place • Boston, MA 02215

Telephone: (617) 735-1930
FAX: (617) 735-1945
diabetes.journal@joslin.harvard.edu

6 March, 2000

Joel F. Habener, MD
Massachusetts General Hospital
Laboratory of Molecular Endocrinology
55 Fruit St.-WEL320
Boston, MA 02114

Re: MS# D00 -048

Dear Dr. Habener:

Your manuscript entitled "The Neural Stem-Cell Protein Nestin Expressed in a Distinct Cell Population Within Pancreatic Islets" has been examined by an outside reviewer and by the editors. Unfortunately, the reviewer was highly critical of the manuscript and, for the reasons outlined in the review, we are unable to accept the paper for publication in *Diabetes*. In looking over the paper, I was struck by Figure 3, in which a duct-like structure is heavily stained for nestin. Because of the thick walls of this structure, I wonder very much if it could be an artery, rather than a pancreatic duct. In any event, your findings are very provocative, but I am afraid that more work will be necessary to strengthen the hypothesis.

I am sure this negative decision will disappoint you, but please keep in mind that we accept only a small number of papers in the "Rapid Publications" category. Certainly, it is in no way meant to adversely influence your submission of these data to be considered as a regular publication in *Diabetes* if you continue your study.

Thank you for submitting your manuscript to *Diabetes*.

Sincerely,

Gordon

Gordon C. Weir, MD
Editor

diabetes COMMENTS TO AUTHORS

Re: MS #D00-048 ("Rapid") 3/6/2000

Title: The Neural Stem-Cell Protein Nestin Expressed in a Distinct Cell Population Within Pancreatic Islets

These comments will be transmitted to the authors. Do not indicate your judgement about acceptibility for publication.

I am compelled by the data and the idea that Nestin could be a marker for the endocrine precursor cells within the islet and the duct, but I think that based on recent data in the literature, that it is critical for the authors to show that these cells express Neurogenin 3. I also believe that it is possible that the authors have actually achieved in culturing islet neurons and not precursor cells at all. It is known that NPY and galanin expressing neurons are found within the islet. It should be ruled out that these cells are not expressing either of these neuropeptides.

I truly believe that if the authors can show that these cells express Neurogenin 3 and that the pseudoislet structures that form in their cultures loose Neurogenin 3 expression and become endocrine that this will be truly important work.

diabetes

JOURNAL OF THE AMERICAN DIABETES ASSOCIATION

Joslin Diabetes Center • One Joslin Place • Boston, MA 02215

Telephone: (617) 735-1930
FAX: (617) 735-1945
diabetes.journal@joslin.harvard.edu

7 June, 2000

Joel F. Habener, MD
Massachusetts General Hospital
Laboratory of Molecular Endocrinology
55 Fruit St.-WEL320
Boston, MA 02114

Re: MS# D00 -048

Dear Dr. Habener:

Your revised manuscript entitled "Stem-Cells Isolated from Pancratic Islets Express Neural Stem Cell Marker Nestin and Differentiate, *Ex Vivo*, into Endocrine Cells" has been examined by the original reviewer, by a new reviewer, and by the editors. Unfortunately, we are still unable to accept the manuscript for publication in its current form Although both reviewers feel that this work may be important, they have raised major concerns. We hope you can address all of the concerns of these reviewers, either with additional experiments or with further clarification.

To resubmit your manuscript, please send four copies of the revised manuscript with the figures, along with four copies of your specific responses to each of the points raised. Also, please include a 3.5-inch diskette labeled with the author's name, manuscript title and number, and the software and hardware used.

Thank you for submitting your work to *Diabetes*.

Sincerely yours,

Gordon

Gordon C. Weir, MD
Editor

diabetes COMMENTS TO AUTHORS

Re: MS #D00 -048
Reviewer #2 6/7/2000

Title: Stem-Cells Isolated from Pancratic Islets Express Neural Stem Cell Marker Nestin and Differentiate, Ex Vivo, into Endocrine Cells

These comments will be transmitted to the authors. Do not indicate your judgement about acceptability for publication.

In this work, Zulewski et al. show that Nestin is expressed in pancreatic islets. They also proposed that Nestin could represent a marker for stem cells that could differentiate into endocrine cells.
This is a very preliminary study. While the working hypothesis is very interesting, neither the data do not support at all the conclusions.

Based on Fig. 1, it is quite clear that Nestin is expressed in the pancreas during embryonic life in rat, and in the islets of Langerhans in the adult pancreas in the rat. This pattern of expression in the adult rat pancreas further confirms data published in rodents in Biochemical and Biophysical Research Communication (Hunziker et al, 271, 116-119; 2000). However, the quality of the Fig.1 can be improved. Fluorescence images obtained with a confocal microscope should be added to clearly demonstrate that nestin-staining is associated with distinct cells. It is also important to show that the nestin-expressing cells are not nerve cells. In the study, an antiserum to galanin was used (page 10 of the MS), but the data are not shown. Such data should be illustrated. Moreover, additional markers for nerve cells should be used to further demonstrate that the nestin-expressing are not nerve cells.
Fig.1, panel E, top right is not totally clear. No ethidium bromide band is seen.

Fig.2 and the corresponding text are also not clear.

First, in Fig2A1 and A2, le cells that grow as a monolayer do not seem phenotypically homogenous. Moreover, while some cells stain positive for nestin, the other stain negative.

How were the doubling times estimated?

The characterization of NIP is not satisfactory. First, RT-PCR is not a proper way to characterize such cells, because it is not clear that those NIPs are clonal and derive from one cell. Thus the PCR signal could be due to any type of contaminating cells. Next, N-CAM is not a good marker for endocrine cells. While it is expressed in endocrine cells in the pancreas during adult life, it is also expressed in nerve and ganglia in the pancreas. Moreover, during embryonic life, it is a strong marker of mesenchymal cells (Esni et al, J Cell Biol, 1999, 144, 325-337). Finally, it is not a specific marker for pancreatic cells. Outside the pancreas, it is expressed in different other cell types.

Concerning Fig3 and the capacity of those NIP cells to differentiate into endocrine tissue.

It is not demonstrated in the present work that NIP can give rise to endocrine cells. Indeed:

1. the IDX1 staining is not convincing..
2. The expression of IDX1 when the cells are grown in the presence of FGF/EGF is not shown.
3. The levels of insulin secretion seem very low and it is not clear that this is not due to contaminating beta cells that were present at the beginning of the culture. In fact, in Fig2A2, it is clear that contaminating insulin-expressing cells are present at the beginning of the culture.
4. The potential importance of betacellulin, activin, HGF have to be studied.

Fig. 4 is fully speculative.

In the discussion section: It has not been demonstrated in Ref 40, that the cells that have been transduced with IDX-1 in the liver are hepatic stem (oval) cells as indicated on page 17.

In conclusion, this is an interesting but very preliminary study that requires further experiments.

diabetes COMMENTS TO AUTHORS

Re: MS #D00-048 (Revised)
Reviewer #1 6/7/2000

Title: Stem-Cells Isolated from Pancratic Islets Express Neural Stem Cell Marker Nestin and Differentiate, *Ex Vivo*, into Endocrine Cells

These comments will be transmitted to the authors. Do not indicate your judgement about acceptibility for publication.

This is a potentially important manuscript for the field of Diabetes and the search for pancreatic stem cells in islets and ducts. The authors have identified what appears to be a unique cell type with non-ductal, non-endocrine morphology, which when given various stimuli appears to show some ability to differentiate into cells secreting endocrine hormones.

Critique:

The identification of potential stem cells in the islets and the ducts is a major finding for those researchers interested in producing β-cells for transplantation. The authors have documented the expression of nestin, a neural stem cell marker, in embryonic as well as adult islet and ducts. These nestin positive cells have a capacity to replicate and differentiate in vitro. Overall, the quality of some of the photomicrographs is quite poor, but the data is well presented. The evidence supporting the major findings in the manuscript needs to be strengthened.

Major Points:

1) In many places in the paper the authors refer to these potential stem cells as pluripotental progenitor cells, while never providing any evidence that these nestin positive cells can become ductal cells (expressing carbonic anhydrase, CFTR, etc.. only cytokeratin 19) or exocrine cells (expressing amylase, trypsin, etc.). Unless the authors can provide evidence that these cells can become ductal, exocrine and endocrine, given the proper stimuli, they should reframe from use of pluripotential. I believe it is possible that these cells do have these properties.
2) Fig. 2B, It would be nice to see nestin staining in the human nestin positive cell cultures. Does their rat antiserum recognize the human?
3) Fig.3a, the authors show a section poorly stained for IDX-1. Do the authors not have any better pictures than these? It would be preferable to show the preculture staining instead of the preimmune serum in Fig. 3b. It would appear that only perhaps 10% of the cells are positive for IDX-1. Have the authors done any cell counting to quantitate what percentage of the cells can become IDX-1 positive. What percentage of the earliest NIP cultures have IDX-1 positive cells? Is there colocalization of nestin and IDX-1?
4) Fig. 3D. The authors show RT-PCR for proglucagon. Was the same performed for insulin? Do the authors have PCR data from precultures for insulin and glucagon?
5) Pg12 paragraph 2. Here the authors state the values obtained for insulin, glucagon and GLP-1 in the media. It is not clear from the methods whether these cultures, which were supplemented with growth factors, also contained the 10% serum that was provided in the early cultures or were these serum free cultures from 72-96 hours. This should be clarified in the methods. If the cultures were with serum, then it is difficult to know if the NIP's really produced these hormones or if they were values obtained from the serum. The values are

Re: MS #D00-048 (Revised)
Reviewer #1

Title: Stem-Cells Isolated from Pancratic Islets Express Neural Stem Cell Marker Nestin and Differentiate, *Ex Vivo*, into Endocrine Cells

These comments will be transmitted to the authors. Do not indicate your judgement about acceptibility for publication.

quite low. I would be much more convinced by seeing nice double staining for nestin and insulin or glucagon or IDX-1 and insulin. It would be quite easy to quantitate the numbers of positive cells in this manner. It would be of great interest to know what the ratio of glucagon to insulin cells produced by the cultures is. If we want to treat diabetes, we would prefer the conditions of cultivation of such cells to make β and not α cells.

6) P18. The authors bring up an important point in paragraph 18 (starting on page 17) about proliferation. Do the authors know if the differentiated endocrine cells in the cultures are post-mitotic? Have they examined BrDu incorporation or MPM-2 or Ki67?
7) It has recently been published that the neural gene Neurogenin-3 is a marker of the precursor cells for the endocrine cells of the pancreas (Nature 400:877-881, 1999; Diabetes 49:163-176, 2000; PNAS 97:1607-11, 2000). It would strengthen the paper greatly for the authors to examine this in their cultures, either by PCR or immunostaining. Transduction Laboratories (www.translab.com) sells a mouse anti-Ngn3 antiserum that works on frozen sections. This could nicely be combined with the rabbit anti-nestin antiserum. Stem cells may not express Ngn-3. We simply don't know.

diabetes

JOURNAL OF THE AMERICAN DIABETES ASSOCIATION

Joslin Diabetes Center • One Joslin Place • Boston, MA 02215

Telephone: (617) 735-1930
FAX: (617) 735-1945
diabetes.journal@joslin.harvard.edu

2 August, 2000

Joel F. Habener, MD
Massachusetts General Hospital
Laboratory of Molecular Endocrinology
55 Fruit St.-WEL320
Boston, MA 02114

Re: MS# D00 -048

Dear Dr. Habener:

Thank you for submitting the second revision of your manuscript entitled "Stem-Cells Isolated from Pancreatic Islets Express Neural Stem Cell Marker Nestin and Differentiate, *Ex Vivo*, into Endocrine Cells." It has been examined by the two reviewers and by the editors. We are left with a difficult problem with your paper, because both reviewers, who have worked hard with this potentially interesting manuscript, still have severe concerns. Both Reviewer #1 and Reviewer #2 have made very clear what will be required for this paper to become acceptable. I have spent a lot of time with this paper also and must agree with their points. With regard to some specific suggestions, there must be a way to obtain better images than 2a. Another problem concerns the hormone results on page 12. The most obvious concern lies with the insulin measurements. Even though the assay is described as being very sensitive, 12-44 pg/ml insulin is a small amount. It seems to me that documenting the presence of insulin in the ILCs must be done more thoroughly so as to be convincing. Thus, more information needs to be provided about the immunoassay and about the number of experiments that were used to draw these conclusions. In addition, statistics are probably indicated in this situation. It should also be possible to show the presence of insulin more convincingly by immunostaining, as well as by PCR.

I hope you can find some way to revise this paper so that the reviewers and editors feel more comfortable with it. As before, to resubmit your manuscript, please send four copies of the revised manuscript with the figures, along with four copies of your specific responses to each of the points raised. Also, please include a 3.5-inch diskette labeled with the author's name, manuscript title and number, and the software and hardware used.

I will be pleased to discuss the matter further with you by telephone.

Sincerely,

Gordon

Gordon C. Weir, MD
Editor

diabetes COMMENTS TO AUTHORS

Re: MS #D00-048RR (2nd revised) 8/2/2000
Reviewer # 1

Title: Stem-Cells Isolated from Pancratic Islets Express Neural Stem Cell Marker Nestin and Differentiate, Ex Vivo, into Endocrine Cells

These comments will be transmitted to the authors. Do not indicate your judgement about acceptability for publication.

Comments to be addressed by the investigators:

Line 20 page 2. Based on these early studies on these cell cultures, the authors should choose to make a less strong statement in the abstract. For example; (NIPs) are a distinct population of cells that *maybe* responsible for.....

Antisera page 6 Authors should mention that the rabbit- anti-nestin antiserum does not recognize the human form in immunocytochemistry or mention this as data not show in the results.

Fig. 1B upper panel: Reviewer 2 commented on the lack of ethidium bromide band in his first review. To clarify this, perhaps the authors should show the original ethidium bromide image for the upper right panel and not the reverse image or vice versa. Listing in the legend the exposure time for the southern blots would also make this figure more clear to the reader, as the very weak upper right panel band has a very strong southern blot signal.

Page 12, Fig 3. This is still the weakest point in the paper and the most important for diabetes. The fact that insulin, glucagon and GLP-1 are detectable by RIA is evidence that hormone production is occurring but I would be much more convinced by seeing an immunostaining to give us an understanding of how many cells become endocrine under these culture conditions. The PCR data for markers NCAM, Cytokeratin 19, proglucagon are nice but since the cells are clearly not 100% clonal (Fig2A2- not all cells stain for nestin), it would be nice to understand which cells are turning off nestin and turning on IDX-1 and insulin and glucagon. Did immunostainings for insulin and glucagon fail to work? If you cannot detect immunoreactive cells by these methods, how do you interpret this? Are no cells cultured now that could be imunnostained?

Figure 3 Legend.. Remove the B in line 4 that refers to the middle panel of A, as this is confusing; with reference to the real panel B appearing two lines below.

diabetes COMMENTS TO AUTHORS

Re: MS #D00-048RR (2nd revised)
Reviewer #2 8/2/2000

Title: Stem-Cells Isolated from Pancratic Islets Express Neural Stem Cell Marker Nestin and Differentiate, Ex Vivo, *into* Endocrine Cells

These comments will be transmitted to the authors. Do not indicate your judgement about acceptability for publication.

This is a second review of a MS by Zulewski et al. I continue to believe that the data do not support the conclusions.
To improve the MS, the authors have to show that:

1. the cells shown in B in Fig. 2, after 2 days in culture stain all positive for Nestin, stain negative for Pdx1 and negative for insulin.
2. that the ILC shown in Fig. 3A stain clearly positive for Pdx1 (the quality of the staining remains quite bad), and positive of insulin.

In that case, the paper will be excellent. Otherwise, it remains an interesting concept without proof.

I do not think that conclusions can be drawn without quantitative data. For example, the meaning of the concentrations of hormones measured in the culture medium of ILC remains obscure if not compared to other time points. What is the content of insulin in ILC?

diabetes

JOURNAL OF THE AMERICAN DIABETES ASSOCIATION

Joslin Diabetes Center • One Joslin Place • Boston, MA 02215

Telephone: (617) 735-1930
FAX: (617) 735-1945
diabetes.journal@joslin.harvard.edu

6 November, 2000

Joel F. Habener, MD
Massachusetts General Hospital
Laboratory of Molecular Endocrinology
55 Fruit St.-WEL320
Boston, MA 02114

Re: MS# D00 -048

Dear Dr. Habener:

Thank you for submitting the third revision of your manuscript entitled "Multipotential Nestin-Positive Stem- Cells Isolated from Adult Pancratic Islets Differentiate, *ex vivo*, into Pancreatic Endocrine, Exocrine and Hepatic Phenotypes." It has been examined by the two original reviewers and by the editors. We would like to accept your very interesting paper for publication in *Diabetes*, but both reviewers have some remaining concerns. Please make whatever additional changes you can to strengthen the paper, and return it to me as soon as possible. I do not intend to send the manuscript back to either of the reviewers, but will review your response and the revisions myself.

As before, when you resubmit your manuscript, please send four copies of the revised manuscript with the figures, along with four copies of your specific responses to each of the points raised. Also, please include a 3.5-inch diskette labeled with the author's name, manuscript title and number, and the software and hardware used.

We look forward to receiving your revised manuscript.

Sincerely,

Gordon

Gordon C. Weir, MD
Editor

diabetes COMMENTS TO AUTHORS

Re: MS #D00-048RRR (revised) 11/6/2000
Reviewer # 1

Title: Multipotential Nestin-Positive Stem- Cells Isolated from Adult Pancratic Islets Differentiate, ex vivo, into Pancreatic Endocrine, Exocrine and Hepatic Phenotypes

These comments will be transmitted to the authors. Do not indicate your judgement about acceptability for publication.

This is the third review of the manuscript by Zulewski et al. Several problems still exist and need to be addressed.

Specific Comments:

Page 12, line 20. It is misleading in Fig 2b that it states a time course of images taken every 24 hours for 18 days when depicted in the figure is only 6 time points. This should be clarified.

Figure 2. This new figure, which was added to the older version of Figure 2, needs to be changed. The quality of the images is quite poor. The insulin image shows nothing at all. You cannot make out anything. The lower panel IDX-1 image should be deleted. In the text it is stated starting on page 13, line 5, ".a small subpopulation of subcloned cells expressing the pancreas specific (also not really a correct term as IDX-1 is also expressed in the duodenum and endocrine cells of the stomach) homeodomain protein IDX-1, possibly reflecting early stages of the differentiation process, however the majority of the cells did not stain for IDX-1". This text refers directly to this image, which is blurry and shows no stained cells.

Page 14, line 10. The word human should be insert before NIP cultures.

Figure 3. The image in B (Upper panel) is much improved over the previous images submitted and the higher magnification insert helps greatly to observe the nuclear staining. I am a little surprised that there is such a weak PCR signal after 35 cycles if this is a representative image and perhaps 5% of the cells express the IDX-1. I think that the middle and lower panels of this figure need to be removed. The middle panel is suppose to show to immunopositive nuclei after treatment with bFGF and EGF then AA and HGF, but the authors don't state anything about what effect this had. Did this increase IDX-1 positive cells? It seems to be what they would like to infer but the image in the middle panel shows nothing conclusive. All one can see is a blurry swirl with perhaps some bright spots. It is quite common to see bright spots that do not correspond to cells when using Cy3 conjugated secondary antiserum, as the dye can aggregate and is very bright. The lower panel adds nothing and is a poor quality image. It can be stated that there was not staining in the control without primary antiserum, without showing this poor image.

Figure legend 3 line 8. The words "after treatment with" should be inserted before bFGF.

Table 1. The legend needs to be clarified. For example, the values 133 and 79 for insulin. Do these values come from a single well of cells (N=1) or from 3 wells that yield one value. If it is only measurement for each, it should be stated so. Also, the paper mentions on page 14, line 21 that

exendin 4 was used and this is not stated in the legend. The data in the table seem quite preliminary.

Figure 4c. It is quite difficult to determine if the values used in the graph (which should have error bars unless it is an n=1, nevertheless, the N value should be stated in the legend) correspond to the values in the table. In the text on page 14 ,line 21, the authors refer to table 1 and Fig 4c for the same treatments but it seems that different numbers are used for the graph and the table. Dish 1 might represent the average of the values for 88-90 days in culture. 321 + 150 gives a value close to what is graphed for dish 1. As for the dish 2 values it might appear this is the 2nd value (66) from the 85-87 days in culture. This really needs to be clarified.

Table 2. The title of the paper suggest that nestin expressing cells can differentiate towards hepatocytes and exocrine cells as well as pancreatic endocrine cells, but the only data provided in support of this is a table that shows no data. It takes nearly as much space in a paper to show a table listing genes as it does to show the PCR bands. If the authors have the data for this they must show it. It is the only evidence supporting that the cells can do what they say.

Page 15, last line. Authors should insert "data not shown" after exocrine acinar tissue, as this is not shown in the figure with which they refer to.

diabetes COMMENTS TO AUTHORS

Re: MS #D00 -048RRR (revised)
Reviewer # 2 11/6/2000

Title: Multipotential Nestin-Positive Stem- Cells Isolated from Adult Pancratic Islets Differentiate, ex vivo, into Pancreatic Endocrine, Exocrine and Hepatic Phenotypes

These comments will be transmitted to the authors. Do not indicate your judgement about acceptability for publication.

This is a third review of a MS by Zulewski et al.
The positive point is that Nestin potentially clearly labels a new cell type in the pancreas. Fig. 2 panel C (Nestin staining) is now clear.
Moreover, indirect evidence suggest that Nestin is a marker of precursor cells.
Based on what I understand, it is very difficult (or impossible) to demonstrate by immunohistochemistry that the nestin-expressing cells can differentiate into insulin-containing cells.

Minor comments.

Abstract, line 2: proliferation has to be added.
Page 4: line 17: IDX-1 seems to be a secreted factor.
Page 19, top: reference 34 and not 28.

diabetes

JOURNAL OF THE AMERICAN DIABETES ASSOCIATION

Joslin Diabetes Center • One Joslin Place • Boston, MA 02215

Telephone: (617) 735-1930
FAX: (617) 735-1945
diabetes.journal@joslin.harvard.edu

15 November, 2000

Joel F. Habener MD
Massachusetts General Hospital
Laboratory of Molecular Endocrinology
55 Fruit St.-WEL320
Boston, MA 02114

Re: MS# D00 -048

Dear Dr. Habener:

I am pleased to inform you that your revised manuscript entitled "Multipotential Nestin-Positive Stem-Cells Isolated from Adult Pancratic Islets Differentiate, *ex vivo*, into Pancreatic Endocrine, Exocrine and Hepatic Phenotypes " has been reviewed and found acceptable for publication in *Diabetes*.

If you need to discuss changes made during copyediting or to ask about journal style, you may contact Christian Kohler, Assistant Managing Editor, at: American Diabetes Association, 1701 North Beauregard Street, Alexandria, Virginia 22311 (phone: 703/299-2083; fax: 703/683-2890; email: ckohler@diabetes.org).

We thank you for submitting your interesting work to *Diabetes* and look forward to receiving additional manuscripts from you. In addition, we hope that you will help us to maintain the high standards of *Diabetes* by continuing to serve as a reviewer when we call upon you in the future.

Sincerely,

Gordon Weir/cr

Gordon C. Weir, MD
Editor

Gordon C. Weir, MD
Editor, Diabetes
Joslin Diabetes Center
One Joslin Place
Boston, MA 02215
(617) 735-2930
Fax (617) 735-2045
diabetes@joslab.harvard.edu

Congratulations on having your manuscript accepted for publication in *Diabetes*.

Listed below are the names of people who can help you with questions about your manuscript:

To inquire about the availability of page proofs:
Christian Kohler, Assistant Managing Editor
phone: (703) 299-2083
fax: (703) 683-2890
email: ckohler@diabetes.org

To inquire about reprints and billing of page charges:
Teresa Everngam, Cadmus Journal Services
phone: (800) 407-9190 ext 299; if calling from outside the U.S.: (410) 819-3914
fax: (410) 820-9765
email: everngamt@cadmus.com

To discuss journal procedures or any other matter pertaining to *Diabetes*:
Aime Ballard, Managing Editor
phone: (703) 299-2088
fax: (703) 683-2890
email: aballard@diabetes.org

Gordon C. Weir, MD
Editor, Diabetes
Joslin Diabetes Center
One Joslin Place
Boston, MA 02215
(617) 735-1930
Fax (617) 735-1945
diabetes@joslab.harvard.edu

Gordon C. Weir, MD, Editor

Date: November 15, 2000

To: Dr. Joel Habener — **Phone:** **Fax:** 726-6954

From: Deborah Moskowitz — **Phone:** 617/ 735-1930
Editorial Office, *Diabetes* — **Fax:** 617/ 735-1945

Number of pages following cover sheet: 3

Message: Please fax back to me, as soon as possible, the attached Color Approval form.

Thank you.

Dr. Henryk Zulewski
Division d'endocrinologie et diabétologie
Hôpitaux Universitaire de Genève
Rue Micheli-du-Crest 24
CH - 1211 Genève 14
Tel. + 4122-372 9305
Fax +4122-372 9309

privé
Avenue de la Grenade 19
CH - 1207 Genève
Tel. 022 - 786 7162
Email : Zulewski@excite.com

18.01.2001

Fax message for

Joel F Habener M.D.
Laboratory of Molecular Endocrinology
Massachusetts General Hospital
Harvard Medical School
Boston, MA 02114
Fax. 617 - 726 6954

Hi Joel ,
please find enclosed my brief remarks concerning the galley proofs.

1. **Authors:**
- All authors (perhaps except Mario) should be identified first with the suffix « 1 » for the Laboratory of Molecular Endocrinology, and than followed by other numbers indicating the actual affiliation.

- Elisabeth is a co-author with equal contribution. This should be marked too with another sign like « # ».

2. Proposition for a short running title
- **Multipotential Stem Cells in Pancreatic Islets**

3. Query H : So far as I recall DPC stands for Diagnostic Products Corporation, Los Angeles, CA

Otherwise, it's a **great paper** and it's a great feeling to see in this format !

With best wishes

DIABETES

THIS IS YOUR REPRINT ORDER FORM AND PRO FORMA INVOICE FOR PUBLICATION FEES

(Please keep a copy of this document for your records.)

Reprint order forms and purchase orders or prepayments must be received 2 weeks before publication either by mail or by fax at 410-820-9765. Please fax Cadmus Reprints at 410-820-9765 by this date if your article contains color and you wish to order reprints.

Author Name Henryk Zulewski
Title of Article Multipotential nestin-positive stem cells isolated from adult...
Issue of Journal March 2001 Manuscript # 582379 Publication Date _____
Number of Pages 13 Color in Article? (Yes) / No (Please Circle)

Please include the journal name and reprint number or manuscript number on your purchase order or other correspondence.

Reprint Costs

Number of reprints ordered 200 $ 920.00
Color in reprints: $70 per 100 copies up to 500 copies $ 140.00
Number of covers ordered _____ $_____
Taxes (Virginia, Washington, DC, or Canadian) $_____
(Add 4.5% VA or 5.75% Washington, DC, sales tax or 7% Canadian GST to the cost of reprints if your order is to be shipped to these locations.)
Add $30 for each add'l ship location $_____

Publication Fees

Page charges (see back) $ 975.00
Number of color figures 2
Color charges: $630 for each color fig. $ 1260.00

Total Amount Due $ 3295.00
(Reprint Cost & Publication Fees)

Shipping Address (cannot ship to P.O. Boxes)

Name Joel.F. Habener, MD
Dept Lab of Molec Endo, MGH
Street 55 Fruit Street, WEL 306
City Boston State MA Zip 02114
Country USA
Quantity 200 Fax 617.726.6954
Phone: Day 617.726.519 Evening _____

Additional Shipping Address*

Name _____
Dept _____
Street _____
City _____ State _____ Zip _____
Country _____
Quantity _____ Fax _____
Phone: Day _____ Evening _____
* Add $30 for each additional shipping address

Ordering Details

Invoice Address
Name
Institution Howard Hughes Medical Institute
Department Accounts Payable
Street 4000 Jones Bridge Road
City Chevy Chase State MD Zip 20815-6789
Country USA
Phone 301.215.8501 Fax 301.215.8525
E-mail Addr harrisa@hhmi.org
Purchase Order No. 009N000878

IT IS THE POLICY OF CADMUS REPRINTS TO ISSUE ONE INVOICE PER ORDER

Credit Card Payment Details

Credit Card: ___ VISA ___ Am. Exp. ___ MasterCard
Card Number _____
Expiration Date _____
Signature _____

Please send your order form and purchase order or prepayment made payable to:

Cadmus Reprints
P.O. Box 751903
Charlotte, NC 28275-1903

Enclosed: Personal Check _____ **Institutional Purchase Order** ✓ **Credit Card Payment Details** _____
Checks must be paid in U.S. dollars and drawn on a U.S. Bank. Credit Cards are processed by Cadmus

Signature _____ Date 1/18/01
Signature is required. By signing this form, the author agrees to accept the responsibility for the payment of reprints and/or all charges described in this document.

Close

rom: "Christian Kohler, SMUF:CKohler@diabetes.org]"
o: 'Haberer, Joel R.'
c:
ubject: dl0048
ent: Wed 1/17/01 10:34 AM
Importance: Normal

Please forward the following message to Dr. Zulewski...

In regard to our telephone conversation yesterday, I have contacted our printer to ensure that you'll be receiving your color proofs by mail. You should be receiving them shortly (by FedEx); however, Figs. 2,3, and 5 will also be in color form. I have instructed the printer that only Figs. 1 and 6 should be in color form. She will be sending you new proofs that will show Figs. 2, 3, and 5 in black and white.

Thank you, and I apologize for the mix-up.

Christian Kohler

Christian S. Kohler
American Diabetes Association
Assistant Managing Editor
Diabetes and Diabetes Care
1701 N. Bearuegard St., Pubs
Alexandria, VA 22314
Ph: 703-299-2083
Fax: 703-683-2890
ckohler@diabetes.org

In vitro cultivation of human islets from expanded ductal tissue

Susan Bonner-Weir*, Monica Taneja, Gordon C. Weir, Krystyna Tatarkiewicz, Ki-Ho Song, Arun Sharma, and John J. O'Neil

Joslin Diabetes Center, Department of Medicine, Harvard Medical School, Boston, MA 02215

Communicated by Paul E. Lacy, Washington University School of Medicine, St. Louis, MO, May 3, 2000 (received for review March 31, 2000)

A major obstacle to successful islet transplantation for both type 1 and 2 diabetes is an inadequate supply of insulin-producing tissue. This need for transplantable human islets has stimulated efforts to expand existing pancreatic islets and/or grow new ones. To test the hypothesis that human adult duct tissue could be expanded and differentiated *in vitro* to form islet cells, digested pancreatic tissue that is normally discarded from eight human islet isolations was cultured under conditions that allowed expansion of the ductal cells as a monolayer whereupon the cells were overlaid with a thin layer of Matrigel. With this manipulation, the monolayer of epithelial cells formed three-dimensional structures of ductal cysts from which 50-to 150-μm diameter islet-like clusters of pancreatic endocrine cells budded. Over 3–4 weeks culture the insulin content per flask increased 10- to 15-fold as the DNA content increased up to 7-fold. The cultivated human islet buds were shown by immunofluorescence to consist of cytokeratin 19-positive duct cells and hormone-positive islet cells. Double staining of insulin and non-β cell hormones in occasional cells indicated immature cells still in the process of differentiation. Insulin secretion studies were done over 24 h in culture. Compared with their basal secretion at 5 mM glucose, cysts/cultivated human islet buds exposed to stimulatory 20 mM glucose had a 2.3-fold increase in secreted insulin. Thus, duct tissue from human pancreas can be expanded in culture and then be directed to differentiate into glucose responsive islet tissue *in vitro*. This approach may provide a potential new source of pancreatic islet cells for transplantation.

One of the main obstacles to successful islet transplantation for both type 1 and 2 diabetes is the limitation of available insulin-producing tissue (1). Only about 3,000 cadaver pancreases become available in the U.S. each year while about 35,000 new cases of type 1 diabetes are diagnosed each year (2) This lack of tissue has given a high priority to efforts to stimulate the growth of new pancreatic islet tissue. Most studies have shown there is limited *in vitro* growth of adult islet cells of any species (3), but several recent reports have found cell proliferation using cultures of adult human islet preparations with extracellular matrix and growth factors (4–8) but these have been associated with loss of insulin production.

From studies on rat pancreatic regeneration (9, 10) we were impressed with the capacity of adult pancreatic duct cells to both expand and differentiate. These data led to the hypothesis (10, 11) that adult duct cells have the potential to lose their specific duct phenotype with rapid proliferation, reverting to multiipotent cells that then can differentiate into islet cells with the appropriate external stimuli. The potential of extracellular matrix as such an external stimulus has been suggested for other cell types (12). Extracellular matrix, in particular laminin, was shown to induce β-casein expression in cultured mammary duct cells (13). Additionally, an overlay of Matrigel, an extracellular matrix preparation, induced the expression of liver-specific genes in clonally expanded hepatocytes (14). Herein we show expansion of human ductal tissue *in vitro* and its subsequent differentiation to islet cells after being overlaid with Matrigel. Over 3–4 weeks culture there was a significant increase in insulin as well as formation of islet-like structures that we have called cultivated human islet buds (CHIBs).

Materials and Methods

Initial Tissue and Culture Conditions. Human islet isolations were performed in the Islet Core Laboratory of the Juvenile Diabetes Foundation Center for Islet Transplantation at Harvard Medical School using the method of Ricordi and coworkers (15). After purification on a Ficoll gradient, the top interface (1.062/1.096 densities) was 50–95% islet with varying amounts of duct and degranulated acinar tissue, the middle interface (1.096/1.11 densities) contained 1–15% islets, duct, and degranulated acini, and the pellet was mostly well granulated acinar tissue with less than 1% islets. In the top and middle layers there were sheets of ductal epithelium from larger ducts whereas the clumps of exocrine cells found in all layers consisted of small intercalated ducts continuing into the acini. From eight collagenase (Liberase, Roche) digested pancreases (donor age 27–59 years), tissue from these layers was cultured in 50 ml of CMRL 1066 (5.6 mM glucose) media plus 10% FBS in Falcon nontreated T-75 flasks (#3012 Becton Dickinson) at 37°C, 5% CO_2. At 1–4 days the nonadherent tissue (both viable and dead) was removed with a media change, and the adherent, or residual, cells were expanded for up to 1 week with additional media changes every 2–3 days. At about 1 week, when most, if not all, adherent cells were in monolayer, the media was changed to 20 ml of serum-free DMEM/F12 (8 mM glucose) medium with 1 g/liter ITS supplement (5 mg/liter insulin + 5 mg/liter transferrin + 5 mg/liter selenium, Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 g/liter BSA, 10 mM nicotinamide, and keratinocyte growth factor (KGF)(10 ng/ml, Roche). We previously had found that DMEM/F12 (8 mM glucose, plus nicotinamide) facilitated growth of rat and pig duct cells *in vitro*. KGF has been reported to be a duct mitogen (16), and we previously had found it to stimulate ductal proliferation *in vitro* without evident changes in cell phenotype. These cells then were grown for about 1–2 weeks until reaching near confluence or forming substantial plaques of epithelial cells. The cells then were layered with Matrigel, a commercial preparation of murine basement membrane (Collaborative Research-Becton Dickinson) as per instructions of supplier for thin layer gel with the exception of an increased gelling time at 37°C. Briefly, the cells were coated with 50 μl Matrigel per cm^2 and allowed to gel overnight before additional media was added. Cell samples were taken at different time points over the course of 6 weeks. Dithizone (diphenylthiocarbozone), which

Abbreviations: CHIBs, cultivated human islet buds; RT-PCR, reverse transcription–PCR; IPF-1, islet promoter factor-1.

*To whom reprint requests should be addressed at: Research Division, Joslin Diabetes Center, 1 Joslin Place, Boston, MA 02215. E-mail: susan.bonner-weir@joslin.harvard.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

stains insulin-containing cells bright red, was used to assess quickly the presence of insulin-producing cells.

Tissue Fixation and Immunochemistry. Monolayer cultures were fixed for 30 min in either 4% (para) formaldehyde (PFA) in 0.1 M phosphate buffer or in Bouin's solution, and then rinsed in the phosphate buffer. Three-dimensional structures (cysts) that formed from these monolayers were harvested by mechanical shearing with a stream of media. Harvested cysts were fixed in PFA for 60 min, enrobed in 2% agar to keep the pellet together through processing and embedding, immersed in the same fixative for another 90 min, washed, and stored in 0.1 M phosphate buffer until routine embedding in paraffin; sections of these were used for immunostaining. Other cysts were fixed in 2.5% glutaraldehyde/0.1 M phosphate buffer for 2 h, washed, and stored in phosphate buffer until being embedded in plastic resin (Araldite, E. F. Fullam, Lanthan, NY) for semithin (1 μm) sections or ultrathin sections for ultrastructural analysis.

Double immunofluorescent staining were done sequentially by using primary antibodies made in different species: Guinea pig anti-human insulin (1:200, Linco Research Immunoassay, St. Charles, MO), rabbit anti-bovine glucagon (1:2,000, kindly donated by M. Appel, University of Massachusetts Medical School, Worcester) and rabbit anti-bovine pancreatic polypeptide (1:3,000, gift of R. E. Chance, Eli Lilly, Indianapolis, IN), rabbit antisynthetic somatostatin (1:300, made in our own laboratory), a mixture of the latter three antibodies (anti-glucagon, -somatostatin, and -pancreatic polypeptide) for identifying the non-β islet cells (17); monoclonal mouse anti-human cytokeratin 19 (CK 19) antibody (1: 100, Dako) (18)or rabbit pancytokeratin (1: 100, Dako); IDX-1 antibody (Hm-253, dilution 1:500 from J. Habener, Massachusetts General Hospital, Boston) (19). The conjugated secondary antibodies used for immunofluorescence were Texas red-conjugated donkey anti-guinea pig IgG, FITC-conjugated donkey anti-rabbit IgG and streptavidin-conjugated FITC (1:100 dilution for all, Jackson ImmunoResearch). Biotinylated horse anti-mouse IgG and normal horse serum were purchased from Vector Laboratories. For cytokeratin and IDX-1 staining of sectioned tissue, antigens were retrieved by microwaving in citrate buffer (three times of 4 min each with the maximum strength of a domestic microwave) (20). Monolayer cultures were incubated for 10–20 min in 0.3% Triton X-100 (Fisher) with 1% lamb serum (GIBCO/BRL) before primary antibody incubation.

Insulin and DNA Content. Harvested cysts or cells removed from flasks by treatment trypsin/EDTA (1× trypsin-EDTA solution, Cellgro, Mediatech Laboratories, Cody, NY; 10–15 min at 37°C) were brought up to 1 ml high-salt buffer (2.15 M NaCl/0.1 M NaH_2PO_4/0.04 M $NaHPO_4$/EDTA, pH 7.4) and then were sonicated three times, 10 sec each at 4–6 W and then stored at −20°C until assayed. Insulin was measured by using a RIA kit for human insulin from Linco. DNA content was measured fluorometerically by using Hoechst 33258 dye as described by DYNA QUANT (Hoefer).

RNA Extraction and Analysis. Total RNA from samples was extracted following manufacturer-suggested protocols using Ultraspec (Biotecx Laboratories, Houston). cDNA synthesis was performed as described (19). PCR was carried out in 50-μl reactions using 3 μl of the diluted cDNA reaction product (corresponding to 20 ng RNA equivalent) as template mixed with 47 μl of PCR mix [1× *Taq* buffer (Promega), 1.5 mM $MgCl_2$ (Promega), 10 pm of each insulin primers (forwards and backwards) (Genosys, The Woodlands, TX), 4 μl of 4:6 ratio of 18S primers/competimers (Classic 18S Internal Standards, Ambion, Austin TX), 80 μM cold dNTPs (GIBCO/BRL), 5 units AmpliTaq Gold DNA polymerase (Perkin–Elmer), and 2.5 μCi [α-^{32}P]dCTP (New England Nuclear)]. Reverse transcription–PCR (RT-PCR) for insulin with 18S ribosomal subunit as internal control was run on the samples. Primers were as follows: human insulin 5′-TCA CAC CTG GTG GAA GCTC; human insulin 3′-ACA ATG CCA CGC TTC TGC (which yield a 179-bp PCR product); and for internal control 18S primers: competimers [Classic 18S Internal Standards (which yield a 488-bp PCR product)]. The thermal cycling protocol began with a denaturing step of 97°C for 10 min, then 19 cycles of 94°C 1 min, 55°C 1 min, 72°C 1 min, and finished with 72°C for 10 min. For human glucagon 5′-ATG AAC GAG GAC AAG CGC; 3′-TTC ACC AGC CAA GCA ATG (which yields a 236-bp product) and human cyclophilin 5′-CCC ACC GTG TTC TTC GAC, 3′-ATC TTC TGC TGG TCT TGCC were used. The reaction volume differed from that above in that 7.5 pmol of each glucagon primer and 25 pmol of each cyclophilin primer were used; the thermal cycle profile was the same except that 23 cycles were used and the annealing temperature was 59°C. Screens were scanned by using a Molecular Dynamics Storm PhosphorImager and reaction products were quantitated with IMAGEQUANT software (Molecular Dynamics). Results are calculated as a percentage of internal standard and presented as mean ± SEM. Reaction conditions were standardized so as to observe linear amplification of PCR products (for both insulin and ribosomal 18S, glucagon and cyclophilin) for different amounts of cDNA (10–50 ng RNA equivalent) and cycle numbers (18–32 cycles). Graded dilutions (1–20%) of a human islet preparation (H99–22, 90% islet purity, 676 ng insulin/μg DNA) were run to establish a standard curve of insulin mRNA to 18 S mRNA and of glucagon mRNA to cyclophilin mRNA. By including two samples from this curve as standards in any other RT-PCR experiment, an estimate of the % islet for a sample could be made.

Insulin Secretion. Three-dimensional structures (cysts/CHIBs) from 1–2 flasks of tissue from pancreas 19, 24, and 25 were harvested at 3–5 weeks culture and washed three times in RPMI (5 mM glucose, 10 mM Hepes, pencillin/streptomycin, 5% FBS). From each flask, 12 aliquots of 40 cysts/CHIBs were incubated in 1.5 ml of the same media in 12-well plates for 4 h at 37°C, the media were removed for measurement of preincubation insulin levels, and fresh media were added for a 24-h incubation. After this 24 h period, media were again removed and measured for basal insulin secretion, and fresh media with either 5 mM or 20 mM glucose were added. At the end of this second 24-h incubation, the final media were removed for measurement with a human insulin RIA kit (Linco).

Results

To promote the attachment of duct cells rather than islet cells, nonsticky culture flasks were used; these flasks have been used to maintain islets in suspension. With pure islet preparations obtained from the top layer of the density gradient, little tissue became adherent even with 7 days culture. It was noted, however, that clumps of nonislet tissue obtained from the top, middle, or pellet layers can adhere to this nonsticky surface starting at about 24 h. It was mainly in the less pure islet preparations that there were adherent cell clumps within 2–4 days. Although there was considerable loss of floating tissue as has been reported for pancreatic acinar tissue in culture (21, 22), the quantity of cell clumps that adhered increased with time. If the nonadherent clumps were removed when the adherent density reached an empirically determined level (covering about 10% of surface), the adherent cells had little to no dithizone staining and included few fibroblasts. Initial samples for insulin and DNA contents were taken at the removal of nonadherent tissue and before the clumps flattened into monolayers. The adherent tissue was only 2.5–24% of the original DNA and 2.5–11% of the original insulin

Table 1. Insulin and DNA content of 75-cm² flask containing cultured human ductal cells

	Original aliquot, 50 ml	Initial adherent	2 wk	3 wk	4 wk
H99-13 (top + middle, 58% islet)					
Insulin, ng	3,200	78.4	—	888	—
DNA, μg	160	3.8	—	22.1	—
I/DNA, ng/μg	20	20.5	—	40.2	—
H99-19 (middle, 5% islet)					
Insulin, ng	nd	70.8	123	344	863
DNA, μg	nd	30.7	39.8	29.8	41
I/DNA, ng/μg		2.3	3.1	11.5	21.1
H99-20 (middle, <5% islet)					
Insulin, ng	1,600	174	—	1,788	2,564
DNA, μg	250	60	—	42.9	46.8
I/DNA, ng/μg	6.4	2.9	—	41.2	54.8

At 2–4 days after islet isolation the majority of the tissue aliquot originally placed into the culture flask was removed, leaving only the tissue adhering to the nontreated surface. Much of the original tissue died as would be expected for acinar tissue. The cell clumps spread to form monolayers; at 2–3 weeks, these monolayers were coated with a thin Matrigel layer. nd, Not determined.

content (Table 1). However, if the nonadherent tissue remained longer in the cultures, both the amount of adherent islet tissue (dithizone positive) and fibroblasts increased (data not shown). With additional time, cells grew from the adherent clumps and formed monolayer plaques of cells with clear epithelial morphology (Fig. 1).

Once the clumps had attached and formed monolayers, the media were changed to serum-free media with added keratinocyte growth factor to favor stimulation of ductal epithelial growth over that of fibroblasts. Over the next 5–10 days the plaques of epithelial cells became nearly confluent. Most of these cells were immunopositive for cytokeratin (results using anti-cytokeratin 19 and anti-pan-cytokeratin were identical), and varying numbers were also islet promoter factor-1 (IPF-1) (PDX-1/IDX-1/STF-1) positive (Fig. 1). The occasional insulin-positive β cells had strong IPF-1 nuclear staining. In addition, many duct cells expressed this transcription factor, both in the nucleus and in the cytoplasm. Scattered cells, both singly and in patches, had cytoplasmic IPF-1 staining with little nuclear staining and again no insulin staining. The large, cytokeratin positive cells in cobblestone patterns are characteristic of pancreatic ductal epithelium. Islets that were included flattened into clusters of small epithelial cells without cytokeratin 19 staining. At the stage of 75–90% confluency, the cultured cells were overlaid with the matrix.

During the first 1–2 weeks with Matrigel, there was movement of the epithelial cells into three-dimensional cystic structures, ranging from 50 to 400 μm in diameter, which often had multiple buds of dithizone-positive cells (Fig. 2). These structures, termed CHIBs, were observed in cultures from all layers and all eight pancreases. The frequency of cysts/CHIBs appeared to depend more on the extent of epithelial confluency than on the layer or pancreas of origin. Control flasks without the matrix overlay produced few, if any, cystic structures but in some preparations some solid spheres formed from the monolayer.

There was significant increase of both the cultured tissue and its insulin content during the 3–6 weeks culture (last 2–3 weeks with Matrigel). Data from the three pancreas from which samples of the full content of 75-cm² flasks were taken initially and at several intermediate time points are shown in Table 1. The insulin/DNA ratio of the starting adherent material (8.2 ± 4.2 ng insulin/μg DNA) was 1–2% that of the islet preparations whether using the mean values from the four purest human islet preparations (top layers) to date (90 ± 2% islet purity, 920 ± 170 ng insulin/μg DNA) or of the purified islets (top layers) from four pancreases of Table 2 (75 ± 4% islet purity, 380 ± 130 ng

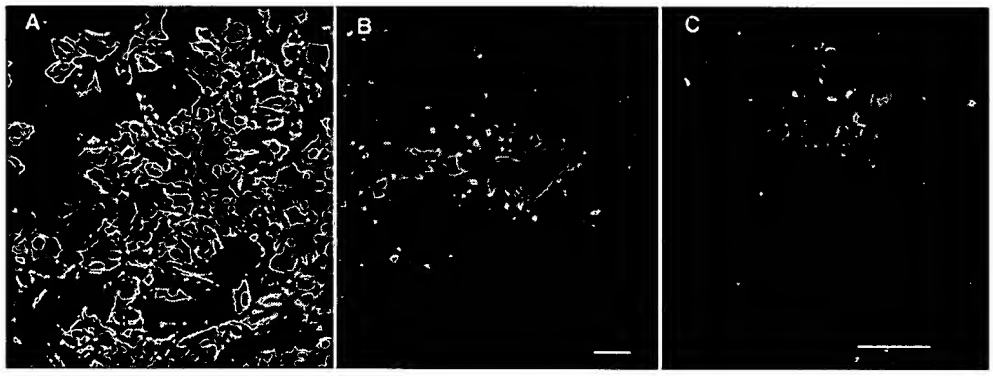


Fig. 1. (*A*) The adherent cells are primarily epithelial cells, immunostained for pan-cytokeratin (FITC green); staining for pan-cytokeratin and cytokeratin 19 were similar. Insulin-positive cells (Texas red) are scattered and infrequent. (*B* and *C*) Double staining of insulin (red) and transcription factor IPF-1 (FITC, green). Besides insulin-producing β cells, many duct cells express this transcription factor, both in the nucleus and in the cytoplasm. In *B* a number of cells express IPF-1 in the nucleus and/or cytoplasm without insulin staining; the field has the same density of cells as *A*. In addition, as in *C*, scattered clumps of cells had cytoplasmic IPF-1 staining with little nuclear staining and no insulin staining. Both *A* and *B* are 7-day cultured tissue of pancreas H99–12 pellet, whereas *C* is 7-day cultured tissue of pancreas H99–10 middle layer. (Magnification bars = 50 μm.)

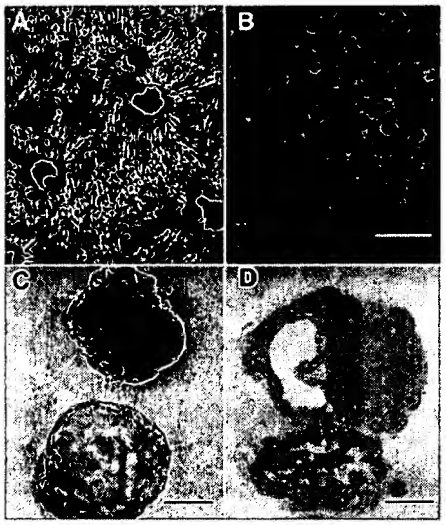


Fig. 2. (*A*) After ducts were overlaid with matrix, three-dimensional structures of ductal cysts with protruding buds of islet tissue (CHIBs) were observed rising from the monolayer lawn of cells. (*B* and *C*) There are variable numbers of dithizone-stained β cells in these harvested cycts/CHIBs; many of the structures are solely cysts whereas other have 50- to 150-μm islet buds. (*D*) The structure of budding islet cells from a cyst is seen in this toluidine blue 1-μm section. (Magnification bar = 500 μm in *B*, 100 μm in *C*, and 50 μm in *D*.)

insulin/μg DNA). Over the 3–4 weeks culture period the insulin/DNA ratio per flask increased, but more importantly the insulin content per flask increased 10- to 15-fold whereas the DNA content increased 0.8- to 7-fold. In contrast, the cultured tissue from the pellet layers showed increases in insulin/DNA ratios but had no increase in insulin and considerable loss of DNA (starting: 63 ± 52 ng insulin, 64.7 ± 13.6 mg DNA $n = 3$, 3–4 wk: 50 ± 10 ng insulin, 24.4 ± 4.2 mg DNA, $n = 3$). However, dithizone-positive CHIBs were formed from these cultures of pellet tissue.

After 2 weeks of matrix overlay, cysts/CHIBs would lift off with the mild agitation of media changes. Others were harvested at the end of the experiment by mechanical shearing with a forceful stream of media. However, this harvesting was imprecise, leaving some CHIBs still attached and lifting off some of the simple ductal cysts as well as some of the remaining monolayer or "lawn." As shown in Table 2, the cysts/CHIBs were enriched in insulin. There was considerable variation in this enrichment with various batches of cysts/CHIBs even from the same pancreas and the same time period, partly because of the imprecision of shearing.

Table 2. Enrichment of insulin content in cysts/CHIBs

Tissue origin		Insulin/DNA (ng/μg), individual samples	Insulin/DNA (ng/μg), mean ± SEM
H99-08	Top	852, 333, 249, 327	440 ± 139
H99-10	Top	48, 66	57
H99-12	Top	149, 61, 149	120 ± 29
H99-13	Top + middle	178, 218, 68	154 ± 45
H99-19	Middle	46, 110	78
H99-20	Middle	110, 152, 94, 27, 74	91 ± 21

Starting at 10 days after Matrigel, some cysts/CHIBs could be collected after becoming dislodged during media changes. Other samples were harvested at the end of an experiment by mechanical shearing. The time of culture for the cysts/CHIB samples was between 5 and 6 weeks (range 27 to 65 days) after isolation, 2–3 weeks after Matrigel (range 10 to 41 days).

Demonstration that the insulin was produced by the tissue and not just adsorbed from the media was confirmed by three approaches: dithizone staining, immunofluorescent staining of the cysts/CHIBs, and semiquantitative RT-PCR for insulin. The CHIBs were composed of cytokeratin 19-positive duct cells and hormone-positive islet cells (Fig. 3). As even suggested by the dithizone-stained samples (as shown already in Fig. 2), the proportion of endocrine tissue in the cysts/CHIBs varied among the different pancreas; many were simple ductal cysts whereas others were cysts with multiple islet buds. The non-β endocrine cells were often equal in proportion to the β cells (Fig. 3 *B–D*). A few cells with double staining for insulin and the non-β cell hormones suggested that some endocrine cells were immature and still in the process of differentiation. Many of the cells within CHIBs had clearly differentiated phenotypes by ultrastructural analysis; both endocrine and mature duct cells were identifiable; however, some cells that had left the ductal epithelium were not granulated (Fig. 4).

The analysis of mRNA by RT-PCR initially showed very low levels of insulin mRNA in the starting material but increases were found as CHIBs developed. Using the standard curve of insulin mRNA/18S mRNA for different dilutions of purified islets, the initial insulin mRNA levels were the equivalent of 0.9 ± 0.4% islet ($n = 6$, all middle layers) whereas, from pancreas H99–20, 5.9% islet at 4 weeks and 5.0% islet in cysts/CHIBs. Similarly, glucagon mRNA levels increased from the initial adherent tissue being equivalent of 1.3 ± 0.7% islet and harvested CHIBs being 14.1 ± 7.2% islet and remaining lawn 4.8 ± 0.8% islet ($n = 3$).

Studies were performed to determine whether the new β cells in these CHIBs could secrete insulin in response to glucose. To address this question, insulin secretion was studied over sequential and parallel 24-h time periods (Fig. 5) in tissue from three pancreases. The data from each pancreas were qualitatively the same. There were no differences in insulin concentration in the basal samples at 5 mM glucose for either the first or second 24-h period (pancreas 19: first, 1.8 ± 0.1 ng/ml, $n = 24$ replicates; second, 1.8 ± 0.1 ng/ml, $n = 12$ replicates). However, in those samples exposed to a stimulatory 20 mM glucose during the second 24-h period, there was a 2.4-fold increase in insulin (pancreas 19: 4.3 ± 0.5 ng/ml, $n = 12$ replicates), demonstrating the glucose responsiveness of the CHIBs.

Discussion

We have been able to expand human duct tissue and then to direct its differentiation to islet endocrine cells *in vitro*. The ability to cultivate human islets *in vitro* from digested pancreatic tissue that is usually discarded after islet isolation opens a new approach for β cell replacement therapy. Human islet isolations yield at best only 400,000–600,000 islets, which means that more than one donor may be required for a successful transplant (2). In the studies reported here, with modest expansion of tissue, insulin content was increased 10- to 15-fold and the endocrine tissue became organized into islet-like structures consisting of β and non-β endocrine cells. These experiments were designed to start with the nonislet ductal tissue with no effort made to salvage islet tissue from the nonislet layers; in fact, because islets rarely adhere to the nonsticky flasks, the conditions did not favor their inclusion. These data provide evidence of the potential to expand and differentiate human duct cells to islet cells, but further optimization of conditions are needed to generate yields of islet tissue that will make an impact on islet transplantation.

Optimization could include further expansion of the ductal tissue or higher efficiency in differentiating cells. Being able to use the pellet layer with higher efficiency would be particularly advantageous because this layer often contains 2–3 times more tissue than the middle layer. It is puzzling why the cultures of pellet layer had the same growth appearance (cobblestone

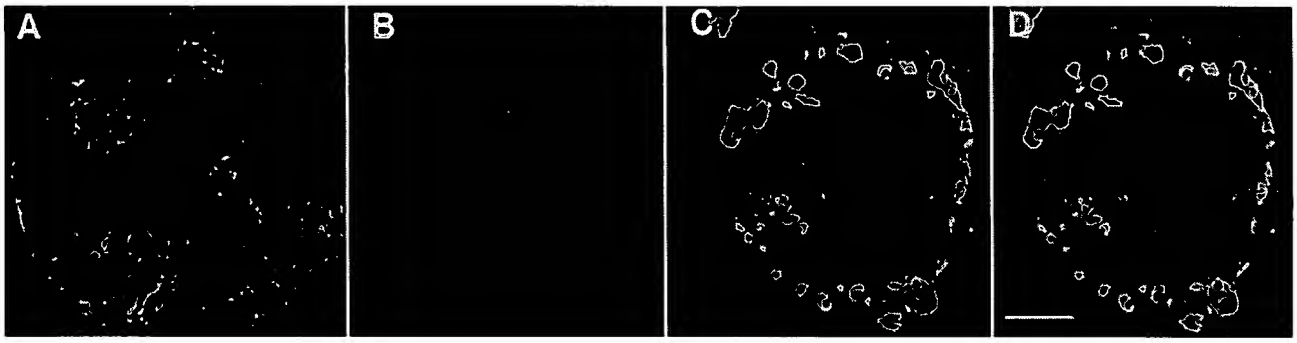


Fig. 3. Double immunostained sections of CHIBs. (*A*) Cytokeratin 19 (FITC, green)-positive duct cells make up most of this CHIB with insulin-positive cells (Texas red) in several islet buds. Another CHIB shown with cell positive for insulin (red in *B* and *D*) and for the non-β cell hormones (glucagon, somatostatin, and pancreatic polypeptide) (green in *C* and *D*); *D* is the overlay of these red and green images. There are a few cells that coexpress both β and non-β cell hormones (yellow in *D*), indicating that some of the cells are immature and still in differentiation. (Magnification bar = 50 μm.)

plaques) and morphogenesis as the middle layer but had no increase in insulin content after application of the Matrigel. The adherent starting tissue was for the most part ductal epithelium, no matter which purification layer was being used. There were some fibroblasts, but the growth conditions favored the epithelial cells. The only noticeable difference in these two layers initially was that the middle had more sheets of duct epithelium from larger ducts whereas the pellet layer had mostly exocrine clumps consisting of small intercalated ducts continuing into acini. Although this difference may be key, the data from rodents suggest that culture of exocrine tissue (the ducts and acini) would result in ductal cultures. Mouse pancreatic exocrine (acinar and ductular) tissue gave rise to epithelial cultures that were indistinguishable from cultures of isolated duct, raising the possibility that acinar cells could dedifferentiate to form duct cells (23, 24). Other studies suggest that between 50% and 95% of the rodent exocrine cells die initially in culture with mainly the ductal cells left to replenish the cultures (21, 22). It is entirely possible that the cells from the smaller ducts/acini have little capacity to differentiate into endocrine cells.

In our study the adherent cells during the early culture period seem to be ductal cells. The large cytokeratin positive cells that form in cobblestone pattern are characteristic of pancreatic ductal epithelium. These large cells often had cytoplasmic and/or weak nuclear staining for the transcription factor IPF-1. In contrast, β cells were small in size, cytokeratin negative and insulin positive by immunostaining, and had strong nuclear staining for IPF-1. Although this transcription factor has been localized mainly to the embryonic duct cells and islet cells, particularly the β and some delta cells (25), we found in the adult rat that recently replicated duct cells also transiently express this protein in the nuclei (11). In these human cultures, the pattern of IPF-1 protein was variable but consistent with recent proliferation of the cells. In contrast, Beattie *et al.* (5) found IPF-1 stained cells only with expanded human islets and not with expanded human duct cells.

There are several lines of evidence supporting that islets are not a major component of the initial adherent cultures. First, there was little dithizone staining of the adherent cells, even from the tissue aliquots from the top layers in which the islets in suspension were strongly stained by dithizone. This is not unexpected because these nontreated flasks had been chosen originally to maintain isolated islets in suspension. In fact, the purer preparations had little adherent tissue; it was when the islet purity was lower (equal or less than 75%) that an appreciable amount of adherent tissue was found. Second, the initial insulin/DNA ratio was less than 2% that of our purest human islet preparations. Because the initial samples for insulin (at 2–4 days) were taken before there was much spreading or replication of the tissue and the suspended islets maintained their insulin content, it is unlikely that adherent islets had lost all of their insulin or became dedifferentiated. This is consistent with the low levels of

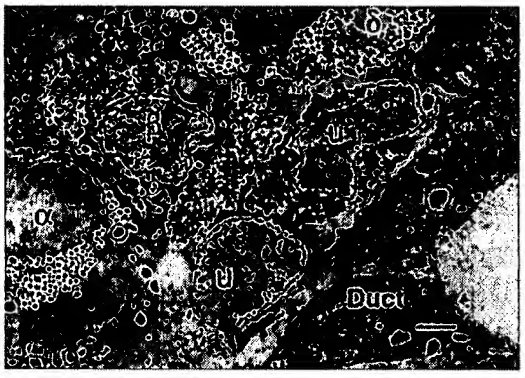


Fig. 4. By ultrastructural analysis of CHIBs, mature and immature phenotypes could be seen. The duct cells (D), with characteristic short stubby microville and apical junctional complexes, line the lumen of a cyst. Adjacent to the ductal epithelium is a row of unidentifiable cells (U) that do not have characteristic granules of islet endocrine cells. β, α, and δ cells are identified by their granules. (Magnification bar = 2 μm.)

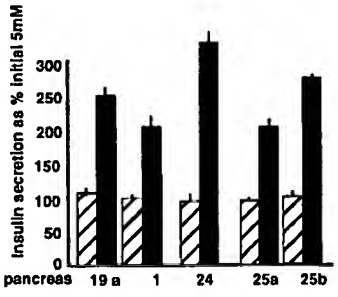


Fig. 5. The cysts/CHIBs are responsive to glucose *in vitro* secretion studies. Forty cysts/CHIBs were incubated in six replicates from each of two groups for three pancreases. After an initial 4-h preincubation, each sample was incubated for 24 h in RPMI with 5 mM glucose for basal secretion determination. The media were replaced with either fresh media with 5 mM (hatched bars) or 20 mM (solid bars) glucose for an additional 24 h. Insulin secretion over the 24-h period was expressed as percent of the same tube at basal. Parallel experiments with middle layer tissue from two flasks of pancreas H99–19 and of H99–25 and one flask from H99–24 are shown. Insulin content was not determined.

insulin mRNA found in these early cultures. To obtain further insight into how many islets might have adhered in the early stages of culture, purified islets of pancreas H99–20 were extracted for insulin and DNA determination, with the finding of 5 ng insulin and 6.5 ng DNA per islet, indicating that each islet consisted of about 930 cells. The amount of insulin contained in the initial adherent tissue of a single flask from this pancreas was 174 ng, which is the equivalent of 35 islets. These 35 islets would contain 228 ng DNA which was 0.4% of the total from the adherent cells of the flask. After 8 days of Matrigel treatment, a flask that started with an identical aliquot of tissue contained 2,560 ng insulin or the equivalent of 512 islets or 7.1% of the final tissue, which was a 15-fold expansion.

Although theoretically it is possible to have increased insulin content and increased insulin-containing cells from replication of the few β cells that were in the initial adherent cell population, we think this is unlikely for several reasons. First, human β cells have been shown to have extremely low replication rate (less than 0.1% labeling for Ki67, a protein present in most cells that are in the cell cycle because it is expressed from mid-G_1 through mitosis); this low level of replication was shown also in late fetal (26) and adult pancreas (27) as well as islet preparations that were cultured with hepatocyte growth factor and on extracellular matrix (8). Second, there is a parallel enrichment of glucagon during the culture as seen by the immunostaining and RT-PCR. Third, the pattern of budding of islet tissue is highly similar to that of *in vivo* neogenesis with the mix of β and non-β endocrine cells with immature endocrine cells as illustrated by both colocalization of islet hormones and ultrastructurally "undifferentiated" cells seen between the duct and endocrine cells. Additionally, the glucose-induced insulin response is immature as one would expect from newly formed islets. Thus, our data strongly favor neogenesis of islet tissue from ductal cells.

Our study differs from previous work in several ways. Most others have started with isolated adult human islets that were expanded on an extracellular matrix substrate (4–8). With expansion as monolayers, human islets were reported to lose their insulin expression but maintain IPF-1 expression (5). With culture in three-dimensional collagen gels human islets also lost insulin expression and endocrine phenotype, becoming duct-like (7). However, using very similar techniques of human islets embedded in three-dimensional collagen gels, Kerr-Conte *et al.* (6) reported proliferation of duct cells, formation of ductal cysts, and then appearance of single endocrine cells in the ductal walls. In the present study, the mainly ductal tissue remaining after islet isolation was expanded and then coated with extracellular matrix to induce differentiation of islet cells. The potential of extracellular matrix to induce differentiation *in vitro* has been shown for other epithelial cell types (12–14). Our technique, which uses Matrigel, a complex matrix with multiple components and growth factors (12), offers the opportunity to dissect the molecular mechanisms involved in the differentiation of the human islet.

The *in vitro* expansion of duct tissue is rapid and extensive, probably because the normal restraints found *in vivo* are removed (10). Because the default pathway of differentiation of embryonic pancreas is thought to be that of islet formation (28), such *in vivo* restraint might be protective and necessary to prevent excessive islet formation that could produce too much insulin and even hypoglycemia. In the present experiments we were able to generate new islet cells from duct cells *in vitro* but the quantities were limited. If the entire middle layer of pancreas H99–20 digest had been cultured, the equivalent of 32,000 new islets could have been generated *in vitro*. This amount would be expected to have little clinical impact although additional differentiation of islet cells from the duct cysts could occur *in vivo*. Despite the limitations at this early stage, these findings raise the tantalizing possibility that this *in vitro* approach, once optimized, might generate meaningful amounts of new human islet tissue from duct cells. This possibility has important implications for making β cell replacement therapy available to a larger number of people with type 1 and 2 diabetes mellitus.

We thank Michael Tenofsky, Jennifer Hollister Lock, and Maggie Merrill for expert technical help; Judy Lebet and Chris Cahill of the Juvenile Diabetes Foundation Center Islet Histology Core; and Richard Parent and Pete O'Neil of the Juvenile Diabetes Foundation Center Islet Isolation Core. The insulin RIAs were done by the Joslin Diabetes Endocrinology Research Center Core. This research was supported by the Juvenile Diabetes Foundation Center for Islet Transplantation at Harvard Medical School and the National Institute of Diabetes and Digestive and Kidney Diseases.

1. Weir, G. C. & Bonner-Weir, S. (1997) *Diabetes* **46**, 1247–1256.
2. Hering, B. J. & Ricordi, C. (1999) *Graft* **2**, 12–27.
3. Brelje, T. C., Scharp, D. W., Lacy, P. E., Ogren, L., Talamantes, F., Robertson, M., Friesen, H. G. & Sorenson, R. L. (1993) *Endocrinology* **132**, 879–887.
4. Beattie, G. M., Cirulli, V., Lopez, A. D. & Hayek, A. (1997) *J. Clin. Endocrinol. Metab.* **82**, 1852–1856.
5. Beattie, G. M., Itkin-Ansari, P., Cirulli, V., Leibowitz, G., Lopez, A. D., Bossie, S., Mally, M. I., Levine, F. & Hayek, A. (1999) *Diabetes* **48**, 1013–1019.
6. Kerr-Conte, J., Pattou, F., Lecomte-Houcke, M., Xia, Y. J., Boilly, B., Proye, C. & Lefebvre, J. (1996) *Diabetes* **45**, 1108–1114.
7. Yuan, S., Rosenberg, L., Paraskevas, S., Agapitos, D. & Duguid, W. P. (1996) *Differentiation* **61**, 67–75.
8. Lefebvre, V., Otonkoski, T., Ustinov, J., Huotari, M., Pipeleers, D. & Bouwens, L. (1998) *Diabetes* **47**, 134–137.
9. Bonner-Weir, S., Baxter, L. A., Schuppin, G. T. & Smith, F. E. (1993) *Diabetes* **42**, 1715–1720.
10. Bonner-Weir, S., Stubbs, M., Reitz, P., Taneja, M. & Smith, F. E. (1997) in *Pancreatic Growth and Regeneration*, ed. Sarvetnick, N. (Karger, New York), pp. 138–153.
11. Cobb, M. H. & Goldsmith, E. J. (1995) *J. Biol. Chem.* **270**, 14843–14846.
12. Streuli, C. H. (1999) *Curr. Opin. Cell. Biol.* **11**, 634–640.
13. Streuli, C. H., Bailey, N. & Bissell, M. J. (1991) *J. Cell. Biol.* **115**, 1383–1395.
14. Block, G. D., Locker, J., Bowen, W. C., Petersen, B. E., Katyal, S., Strom, S. C., Riley, T., Howard, T. A. & Michalopoulos, G. K. (1996) *J. Cell. Biol.* **132**, 1133–1149.
15. Linetsky, E., Bottino, R., Lehmann, R., Alejandro, R., Inveradi, L. & Ricordi, C. (1997) *Diabetes* **46**, 1120–1123.
16. Yi, E., Yin, S., Harclerode, D. L., Bedoya, A., Bikhazi, N. B., Housley, R. M., Aukerman, S. L., Morris, C. F., Pierce, G. F. & Ulich, T. R. (1994) *Am. J. Pathol.* **145**, 80–85.
17. Montana, E., Bonner-Weir, S. & Weir, G. C. (1993) *J. Clin. Invest.* **91**, 780–787.
18. Bouwens, L., Braet, F. & Heimberg, H. (1995) *J. Histochem. Cytochem.* **43**, 245–253.
19. Sharma, A., Zangen, D. H., Reitz, P., Taneja, M., Lissauer, M. E., Miller, C. P., Weir, G. C., Habener, J. F. & Bonner-Weir, S. (1999) *Diabetes* **48**, 507–513.
20. Leong, A. S-Y. & Milios, J. (1993) *Appl. Immunohistochem.* **1**, 267–274.
21. Logsdon, C. D. & Williams, J. A. (1986) *Am. J. Physiol.* **250**, G440–G447.
22. Elsasser, H. P., Lutcke, H. & Kern, H. F. (1986) *The Exocrine Pancreas: Biology, Pathobiology, and Disease* (Raven, New York), pp. 45–53.
23. Githens, S., Schexnayder, J. A., Moses, R. L., Denning, G. M., Smith, J. J. & Frazier, M. L. (1994) *In Vitro Cell. Dev. Biol.* **30**, 622–635.
24. Githens, S., Schexnayder, J. A., Desai, K. & Patke, C. L. (1989) *In Vitro Cell. Dev. Biol.* **25**, 679–686.
25. Guz, Y., Montminy, M. R., Stein, R., Leonard, J., Gamer, L. W., Wright, C. V. E. & Teitelman, G. (1995) *Development (Cambridge, U.K.)* **121**, 11–18.
26. Bouwens, L., Lu, W. G. & Krijger, R. D. (1997) *Diabetologia* **40**, 398–404.
27. Bouwens, L. & Pipeleers, D. G. (1998) *Diabetologia* **41**, 629–633.
28. Gittes, G. K., Galante, P. E., Hanahan, D., Rutter, W. J. & Debase, H. T. (1996) *Development (Cambridge, U.K.)* **122**, 439–447.

nature biotechnology

Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages

Raewyn M Seaberg[1,6], Simon R Smukler[1,6], Timothy J Kieffer[2], Grigori Enikolopov[3], Zeenat Asghar[4], Michael B Wheeler[4], Gregory Korbutt[5] & Derek van der Kooy[1]

The clonal isolation of putative adult pancreatic precursors has been an elusive goal of researchers seeking to develop cell replacement strategies for diabetes. We report the clonal identification of multipotent precursor cells from the adult mouse pancreas. The application of a serum-free, colony-forming assay to pancreatic cells enabled the identification of precursors from pancreatic islet and ductal populations. These cells proliferate *in vitro* to form clonal colonies that coexpress neural and pancreatic precursor markers. Upon differentiation, individual clonal colonies produce distinct populations of neurons and glial cells, pancreatic endocrine β-, α- and δ-cells, and pancreatic exocrine and stellate cells. Moreover, the newly generated β-like cells demonstrate glucose-dependent Ca^{2+} responsiveness and insulin release. Pancreas colonies do not express markers of embryonic stem cells, nor genes suggestive of mesodermal or neural crest origins. These cells represent a previously unidentified adult intrinsic pancreatic precursor population and are a promising candidate for cell-based therapeutic strategies.

There is considerable excitement and controversy about the nature of putative precursor cells in the adult mammalian pancreas[1], largely because of their potential as a source of β-cells for transplantation in the treatment of diabetes. The focus of many studies has been the development of cell culture strategies to expand insulin-producing β-cells[2]. A number of issues relating to the nature of pancreatic precursors have not been resolved, including whether they reside in the islets of the endocrine pancreas or ducts of the exocrine pancreas, their capacity for self-renewal and their full lineage potential. One obstacle to investigating these issues is that there have been no reports of the clonal isolation and proliferation of single adult pancreatic precursor cells.

Some evidence suggests that pancreatic cells[3], and specifically precursors[4,5], may express nestin, an intermediate filament protein that is a sensitive but not specific marker of neural precursor cells[6]. Although these studies have provided evidence for a proliferative nestin-expressing pancreatic cell, these cells have remained incompletely characterized because they were cultured in large, nonclonal populations[4]. We sought to identify single proliferative cells from the adult pancreas and characterize them in terms of their gene expression, their lineage potential and their possible developmental origins. We further sought to explore the relationship between the $nestin^+$ cells from the adult brain and adult pancreas.

Here we report the rigorous clonal isolation of multipotential precursor cells from the adult pancreas. These precursors proliferate in the serum-free media conditions routinely used for neural stem cell culture and form floating clonal colonies. Such pancreas colonies arise from precursors present in both islet- and duct-derived populations, and from both $nestin^+$ and $nestin^-$ cell fractions. Intriguingly, clonal pancreas colonies express markers characteristic of both pancreatic and neural precursors. Upon differentiation, clonal pancreas colonies generate multiple types of neural progeny including mature neurons. Surprisingly, pancreas colonies generate a significantly higher proportion of neurons than do adult brain-derived clonal neurospheres. In addition, pancreas colonies generate islet endocrine cell types including mature pancreatic insulin-producing β-like cells, glucagon-producing α-cells and somatostatin-producing δ-cells. The β-like cells generated *de novo* are functional in that they exhibit glucose-dependent Ca^{2+} responsiveness and insulin release. Pancreas colonies also generate acinar cells characteristic of the exocrine pancreas and pancreatic stellate cells. Thus, they are able to generate multiple neuroectodermal and endodermal cell types. In light of this result, we have termed these cells pancreas-derived multipotent precursors (PMPs). To our knowledge, there have been no previous reports of a robust adult somatic cell population from the pancreas that is capable of reliably and reproducibly generating clonal progeny characteristic of both endocrine and exocrine pancreatic lineages, and indeed progeny characteristic of more than one primary germ layer.

[1]Department of Medical Genetics and Microbiology, University of Toronto, Toronto M5S 1A8, Canada. [2]Department of Physiology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada. [3]Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, USA. [4]Department of Physiology, University of Toronto, Toronto M5S 1A8, Canada. [5]Department of Surgery & Department of Physiology, University of Alberta, Edmonton, Alberta T6G 2N8, Canada. [6]These authors contributed equally to this work. Correspondence should be addressed to R.M.S. (raewyn.seaberg@utoronto.ca).

Published online 22 August 2004; doi:10.1038/nbt1004

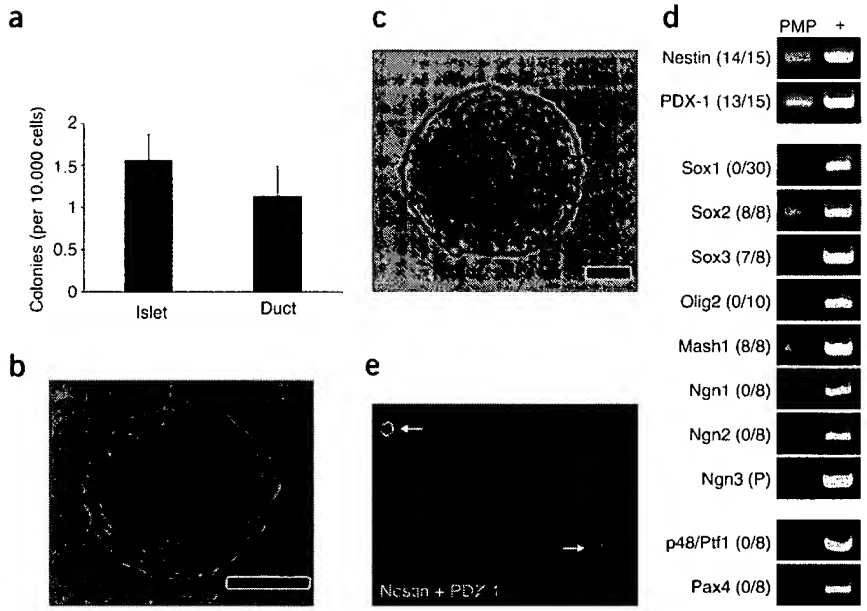


Figure 1 PMP colonies are formed from progenitors present in adult pancreatic islet and duct cell isolates, and express markers characteristic of both neural and pancreatic precursors. (**a**) The frequency of PMP colonies from pancreatic islet and duct cell isolates is similar. The data are expressed as the mean number of colonies (+s.e.m.; $n = 14$ independent experiments) formed per 10,000 cells plated. Islet and ductal cell isolates do not contain significantly different numbers of PMPs ($P > 0.05$). (**b**) Light micrograph of a PMP colony. Scale bar, 50 μm. (**c**) Light micrograph of a neurosphere. Scale bar, 50 μm. (**d**) RT-PCR for neural and pancreatic precursor markers. The numbers on the left represent the number of individual PMP colonies that expressed the corresponding mRNA out of the total number of colonies tested by RT-PCR analysis. Only single colony RNA isolates that were found to express β-actin were considered. Note that positive control (+) bands (see **Supplementary Methods** online for a complete list of tissue positive controls) appear brighter because of the greater amount of starting RNA in comparison to single PMP colonies. Ngn3 was not expressed at detectable levels in individual PMP colonies. However, Ngn3 mRNA was detected in a sample of five pooled (P) PMP colonies, suggesting that it is present in differentiated PMP colonies but perhaps at low levels. (**e**) Single cells from dissociated PMP colonies coexpress PDX-1 (red) and nestin (green) as seen by immunostaining. Note that the nucleus in this fluorescence micrograph is labeled with both DAPI (blue) and PDX-1, giving it a pink appearance. The white arrows indicate double-positive cells.

RESULTS

Colonies arise clonally from single islet and ductal cells

To determine whether cells isolated from adult pancreatic islets and ductal tissue would proliferate *in vitro*, we used defined serum-free media conditions typical for the isolation of brain-derived neural stem cells. In these conditions, neural stem cells clonally proliferate to form floating cell colonies called neurospheres[7]. Pancreatic islets and ductal tissue were separately dissociated into single cells and plated at low density in the serum-free medium containing epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2).

By 7 d *in vitro*, floating colonies resembling neurospheres formed in islet and duct cultures (**Fig. 1**). There was no significant difference in the number of colonies formed from islet (1/6,410 cells) and ductal (1/8,850 cells) cells ($P > 0.05$) (**Fig. 1a**). Indeed, throughout the following analyses there were no differences noted between islet- and ductal-derived precursor colonies; henceforth, they will be referred to collectively as pancreas colonies or (based on the following analyses) PMP colonies. Although the PMP colonies were morphologically similar to neurospheres (**Fig. 1c**), on average they were smaller in diameter (104 ± 8.6 μm PMP colony compared with 263 ± 7.7 μm neurosphere[8]) (**Fig. 1b**) and did not increase substantially in size upon lengthening of the culture period. Each PMP colony contained 2,000–10,000 cells.

Experiments were done to confirm that the PMP colonies were arising from individual cells and not by multicellular aggregation. First, we conducted mixing experiments in which equal proportions of wild-type cells (white, unmarked) and cells marked with green fluorescent protein (GFP^+) from animals constitutively expressing GFP[9] were dissociated and plated together at a final density of 20 cells μl^{-1}. The resulting colonies were assayed for the number of white, green and mixed colonies. Mixed colonies are indicative of cellular aggregation. This type of analysis has been used to demonstrate the clonal derivation of other precursor colonies, including brain-derived neural stem cells[10], retinal stem cells[11] and inner ear stem cells[12]. Of the 114 PMP colonies assayed, some were wholly unmarked, some were apparently wholly GFP^+ and none were mixed. These data indicate that PMP colonies do not arise by aggregation when pancreas cells are plated at 20 cells μl^{-1} or lower, but rather by the proliferation of single pancreatic precursors.

To confirm the clonality of PMP colonies more rigorously, we carried out single cell analyses. Cells were plated at a density of 0.05 cells μl^{-1} in 96-well plates. At the outset of the culture period, wells were assayed for the presence of single cells, and only these wells were included in further analysis. Altogether, 15,335 single cells were followed, of which five (0.03%) formed colonies. This percentage of colony-forming cells is similar to the observed ~0.02% (**Fig. 1a**) of cells that form colonies in routine culture conditions of 20 cells μl^{-1} (a density 400-fold greater than that used for the single cell analysis). These data indicate that the PMP colonies arise from the clonal proliferation of single cells and not from cellular aggregation. All subsequent analyses were carried out on clonal PMP colonies generated from the proliferation of a single cell.

Single colonies express neural and pancreatic precursor markers

The pancreatic transcription factor PDX-1 is one of the earliest genes expressed in the developing[13,14] and regenerating[15] pancreas. $PDX\text{-}1^+$ cells generate both exocrine and endocrine compartments of the pancreas during development[14]. Other markers of pancreatic precursors include p48/Ptf1, Pax4 and Ngn3[16]. Ngn3 is also expressed in neural precursors, as are Ngn1 and Ngn2[17], nestin[6], Sox1-3[18], Mash-1[19] and Olig2[20]. To determine whether PMPs expressed markers more characteristic of neural or pancreatic precursors, we carried out RT-PCR analysis of these markers on single colonies. Nearly all PMP colonies expressed both nestin (14/15) and PDX-1 (13/15) (**Fig. 1d** and **Supplementary Table 1** neurosphere comparison online). Also, single cells from acutely dissociated PMP colonies (i.e., cells from dissociated

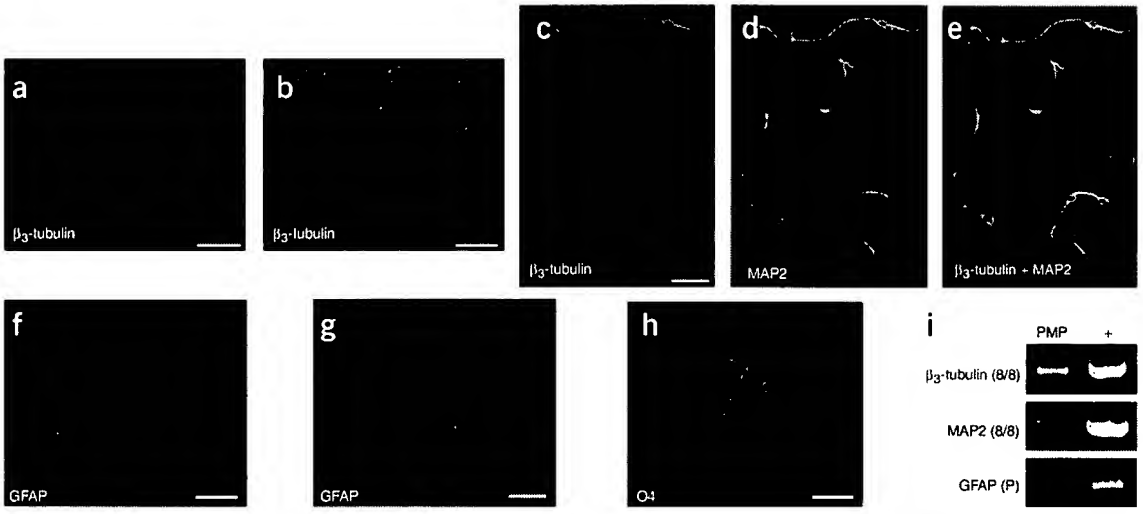


Figure 2 PMP colonies generate all three major neural cell lineages. (**a**,**b**) When individual PMP colonies were differentiated, they were found to generate β_3-tubulin$^+$ neurons (red), occasionally forming large neuronal networks as shown in **b**. Scale bars: 50 μm, **a**; 200 μm, **b**. (**c**–**e**) β_3-tubulin$^+$ neurons that were generated by PMPs (**c**) coexpressed the more mature neuronal marker MAP2 (green) (**d**, and overlay **e**), thus confirming their neuronal identity. Scale bar, 50 μm. (**f**,**g**) PMPs generated GFAP$^+$ astrocytes (green). Scale bars, 20 μm. (**h**) O4$^+$ oligodendrocytes also were generated by PMP colonies (green). Scale bar, 20 μm. All nuclei were counterstained with DAPI (blue) for purposes of quantification. Refer to **Table 1** for relative proportions of each neural cell type produced by PMPs. (**i**) RT-PCR analyses confirm the presence of mRNA for neuronal and glial makers. Individual differentiated clonal PMP colonies all expressed detectable levels of β_3-tubulin and MAP2, but not GFAP. However, GFAP mRNA was detected in a sample of five pooled (P) PMP colonies, suggesting that it is present in differentiated PMP colonies but at lower levels. This is in accordance with the relatively lower percentages of glial than neuronal progeny determined by immunocytochemistry (**Table 1**). Only single colony RNA isolates that were found to express β-actin were considered. Note that positive control (+) bands appear brighter because of the greater amount of starting RNA in comparison to single PMP colonies.

PMP colonies were only very briefly cultured to allow attachment before fixation) coexpressed PDX-1 and nestin (**Fig. 1e**). Individual PMP colonies also expressed Sox2, Sox3, Mash-1 and Ngn3 (but not Pax4, p48/Ptf1, Olig2, Sox1, Ngn1 and Ngn2) (**Fig. 1d**). This indicated that clonal PMP colonies expressed markers characteristic of both pancreatic and neural precursors and hinted that the PMP cell might be a novel precursor that could generate both neural and pancreatic progeny.

Pancreatic precursors generate multiple neural lineages

To determine whether PMP colonies would generate neural or pancreatic progeny, we removed individual clonal PMP colonies from mitogen-containing media, replated them on an adherent substrate and differentiated them for 7 d. PMP colonies from both islet and duct cultures generated β_3-tubulin$^+$ neurons, GFAP$^+$ astrocytes and O4$^+$ oligodendrocytes (**Fig. 2**). These cell types are routinely generated by neural stem cells when brain-derived neurospheres are differentiated in this manner. Under identical differentiation conditions, the PMP colonies generated very different proportions of these cell types compared with brain-derived neurospheres (**Table 1**). For example, neurospheres generated a higher proportion of GFAP$^+$ astrocytes (84.2% ± 1.4%) than did PMP colonies (7.4% ± 1.3%), although neurospheres and PMP colonies generated similar numbers of O4$^+$ oligodendrocytes (4.3% ± 1.7% and 2.4% ± 0.7%, respectively). Notably, PMP colonies generated a significantly greater proportion of neurons (26.4% ± 3.8%) than did brain-derived neurospheres (3.7% ± 0.6%). In rare cases, differentiated PMPs formed colonies consisting primarily of neurons with extensive networks of neuronal processes (**Fig. 2b**). Neurons were also colabeled with MAP2 (**Fig. 2c–e**), a mature neuronal marker that confirms the identity of these cells. The detection of β_3-tubulin and MAP2 protein was critical because islet cells in monolayer culture can sometimes extend short neurite-like processes. However, in the present study, cells immunopositive for any endocrine or exocrine pancreatic marker were never found to extend neurites. In addition to the morphological and immunocytochemical evidence, the presence of mature neural lineages in the clonal differentiated PMP cultures was confirmed by RT-PCR of individual colonies for β_3-tubulin and MAP2 (**Fig. 2i**). GFAP was undetectable in single colonies but was detected when five colonies were pooled, suggesting that it is present in differentiated PMP colonies but at lower levels. This is in accordance with the relative percentages of glial and neuronal progeny as determined by immunocytochemistry (**Table 1**).

Pancreatic precursors generate cells with β-cell properties

Surprisingly, the same clonal PMP colonies that generated neural progeny (n = 100 individual clonal PMP colonies) also generated insulin$^+$ and C-peptide$^+$ (a cleavage product of the insulin prohormone released during insulin production) pancreatic β-like cells. This result was found for both islet (**Fig. 3a**) and ductal (**Fig. 3b**) clonal colonies by the identification of β_3-tubulin$^+$ and either insulin$^+$ or C-peptide$^+$ cells within the same single clonal colony, indicating that the original colony-forming PMP cells were multipotential for both neural and pancreatic lineages. It was important to confirm that the insulin$^+$ cells identified in differentiated PMP cultures were β-cells and not an unrelated cell type concentrating insulin from the culture medium[21]. To further confirm that the insulin$^+$ cells had the characteristics of β-cells, we carried out a series of double-labeling experiments using antibodies against C-peptide. Single cells from differentiated PMP colonies coexpressed insulin or C-peptide and PDX-1, a transcription factor expressed by mature β-cells[15] (**Fig. 3c,d**). All single C-peptide$^+$ cells coexpressed PDX-1. Further, single cells were colabeled with C-peptide and insulin (**Fig. 3e**) or C-peptide and Glut2 (**Fig. 3f**). Every single insulin$^+$ cell coexpressed C-peptide$^+$, and every C-peptide$^+$ cell coexpressed Glut2, demonstrating that insulin immunoreactivity

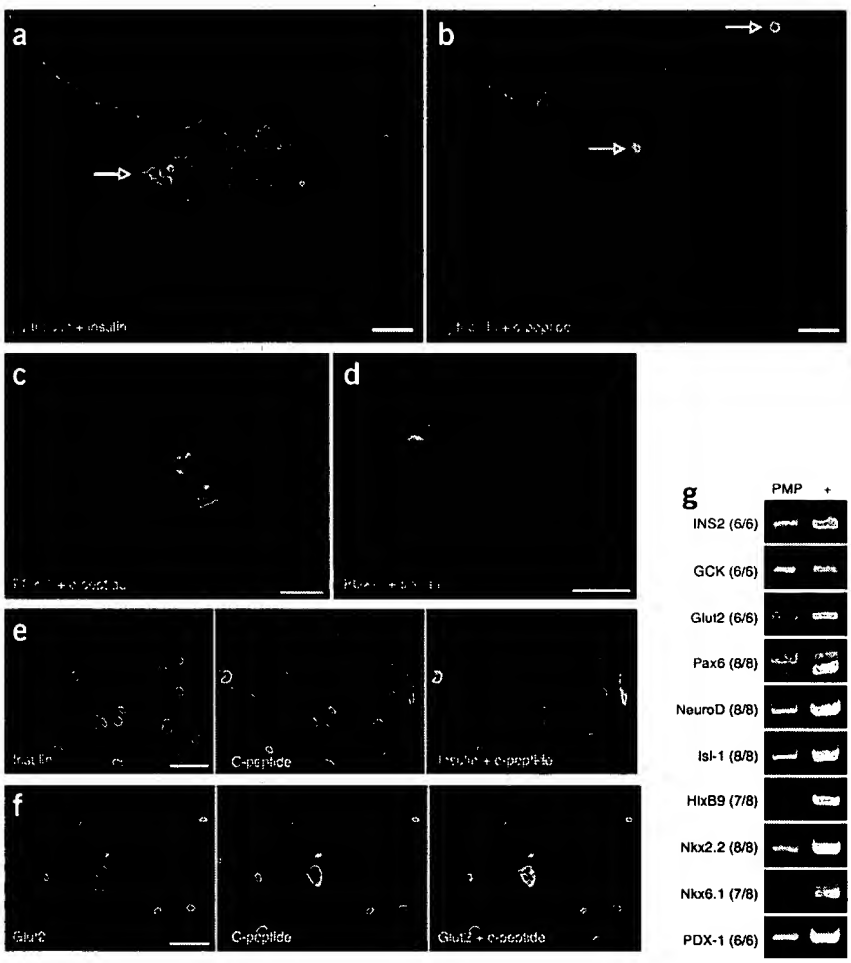


Figure 3 Progeny from two distinct embryonic primary germ layers are generated by single, clonally derived PMPs that are present in islet and ductal cell isolates. (**a**,**b**) Upon differentiation, single islet- (**a**) and ductal- (**b**) derived PMP colonies generated both β_3-tubulin$^+$ neurons (red) and insulin$^+$ or C-peptide$^+$ β-cells (green). Note that although only one combination of β_3-tubulin and insulin or C-peptide is shown for each of islet and ductal PMP colonies, both islet and ductal PMP colonies contained insulin$^+$ and C-peptide$^+$ cells in combination with β_3-tubulin. The white arrows indicate insulin$^+$ and C-peptide$^+$ cells. Scale bars, 50 μm. (**c**,**d**) To confirm that the insulin$^+$ cells represented β-cells and were generating insulin protein *de novo*, differentiated colonies were colabeled with antibodies against PDX-1 and C-peptide (**c**) or insulin (**d**). These micrographs illustrate single colonies with cells positive for both PDX-1 (red) and C-peptide or insulin (green). Scale bars, 25 μm. (**e**,**f**) Insulin$^+$ cells (red) all coexpress C-peptide (green) as illustrated by the merged field (yellow) (**e**) and C-peptide$^+$ cells (green) all coexpress Glut2 (red) as shown in the merged field (yellow) (**f**). Scale bars, 50 μm. Although only one example of each is illustrated, both islet- and ductal-derived PMP colony progeny exhibited these patterns. In all micrographs nuclei have been counterstained with DAPI for purposes of quantification. Note that in **c** and **d**, nuclei appear pink because of the colocalization of DAPI and PDX-1. Refer to **Table 1** for the proportion of cells with β-cell characteristics produced by single PMPs. (**g**) RT-PCR analyses confirm that single clonal differentiated PMP colonies express many characteristic islet/β-cell markers. Only single-colony RNA isolates that were found to express β-actin were considered. Note that positive control (+) bands appear brighter because of the greater amount of starting RNA in comparison to single PMP colonies.

is not a consequence of uptake from the culture medium. Colabeled cells expressing both C-peptide and Pax6 were also observed (see **Supplementary Fig. 1** online).

To determine whether differentiated PMP colonies expressed other characteristic markers of β-cells/islet cells, we carried out RT-PCR on PMP colonies for insulin II (Ins2), glucokinase (GCK), Glut2, Pax6, Beta2/NeuroD, Hlxb9, Isl-1, Nkx2.2, Nkx6.1 and PDX-1. Single clonal differentiated PMP colonies expressed these genes (**Fig. 3g**). Together, these data demonstrate that PMPs generate cells with β-cell characteristics upon differentiation.

PMP-generated cells show glucose-dependent Ca^{2+} responses

To determine whether the β-like cells in single clonal differentiated PMP cultures are functional, we carried out intracellular Ca^{2+} ($[Ca^{2+}]_i$) imaging studies. Single cells were identified for $[Ca^{2+}]_i$ imaging by prior infection with AdRIP2EYFP, an adenovirus in which expression of enhanced yellow fluorescent protein (EYFP) is controlled by the rat insulin II gene promoter (RIP2). Within the islet, EYFP expression is specific to insulin$^+$ β-cells[22], so identification of EYFP$^+$ cells in these differentiated PMP cultures further supports their β-cell identity (**Fig. 4a**,**b**). EYFP$^+$ cells from both islet- and ductal-derived PMP cultures exhibited a $[Ca^{2+}]_i$ response to stimulation by glucose (**Fig. 4c**,**d**). This response was augmented by the physiological secretagogue glucagon-like peptide-1 (GLP-1), which stimulates β-cells in a glucose-dependent manner[23], or tetraethylammonium (TEA), a compound that inhibits delayed rectifier K^+ currents and potentiates the glucose-stimulated insulin response[24]. Further, this $[Ca^{2+}]_i$ response was abolished by the addition of the voltage-dependent Ca^{2+} channel blocker verapamil. These results demonstrate that the EYFP$^+$ cells present in cultures of differentiated PMP colonies are glucose responsive.

PMP-generated cells show glucose-dependent insulin release

To further characterize the PMP-derived glucose-responsive cells, we determined insulin content and compared it to primary islet cells. PMP-derived cells contained 40.2 ± 10.9 ng of insulin/μg of DNA, and primary islet cells contained 116.9 ± 22.6 ng of insulin/μg of DNA. Basal insulin secretion over 1.5 h from differentiated PMP-derived cells was found to be 56.5 ± 7.1 pg of insulin/differentiated PMP colony. PMP-derived cells demonstrated increased insulin secretion in response to glucose alone or to glucose plus GLP-1 or plus TEA (**Fig. 4e**,**f**). These cells also secreted insulin in response to carbachol, a cholinergic agonist capable of stimulating insulin release even under low glucose conditions[25]. Verapamil abolished glucose-stimulated insulin release to basal levels. These data clearly indicate that there are cells present in cultures of differentiated PMP colonies that exhibit the functional properties of β-cells.

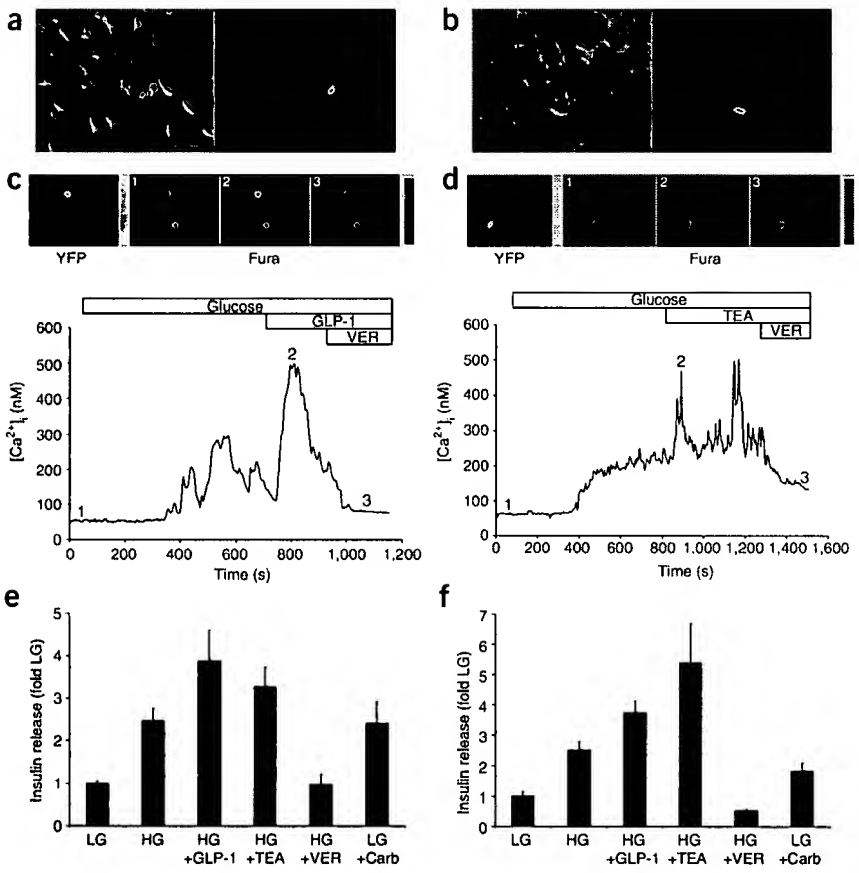


Figure 4 Insulin$^+$ cells generated *de novo* from PMPs demonstrate glucose-stimulated Ca^{2+} responses and glucose-stimulated insulin release. (**a**,**b**) Bright-field and fluorescence micrographs demonstrating YFP$^+$ cells from AdRIP2EYFP-infected islet- (**a**) and ductal- (**b**) derived differentiated PMP colonies. Scale bars, 50 μm. (**c**,**d**) Calcium traces for islet- (**c**) and ductal- (**d**) derived PMP colonies demonstrating glucose-stimulated $[Ca^{2+}]_i$ responses, which were augmented by the addition of either GLP-1 or TEA, respectively. The addition of the voltage-dependent Ca^{2+} channel blocker verapamil (VER) returned the $[Ca^{2+}]_i$ to basal levels. Shown above the Ca^{2+} trace are fluorescence micrographs of YFP$^+$ cells and the ratiometric Fura images (pseudocolored according to the scale shown to the right) corresponding to the numbered time points on the trace. Note that in (**c**), the YFP$^-$ cell does not demonstrate a glucose response. These Ca^{2+} traces are representative of at least five independent experiments. Note that GLP-1 and TEA produced similar responses in both islet- and ductal-derived PMP progeny, although only one example is depicted for each in the $[Ca^{2+}]_i$ traces shown. (**e**,**f**) Demonstration of increased insulin release by islet- (**e**) and ductal- (**f**) derived PMP colonies in response to high glucose (HG) alone or with the addition of GLP-1, TEA or to Carbachol (Carb) alone. The addition of verapamil (VER) abolished the glucose-stimulated insulin release. These data were generated from three to four independent experiments.

Pancreatic precursors generate multiple pancreatic cell types

To determine whether PMPs could generate other subtypes of islet endocrine cells, we tested differentiated clonal PMP colonies for the presence of α-cells and δ-cells using antibodies specific for glucagon and somatostatin, respectively. We found that both α-cells (6.3% ± 2.0%) and δ-cells (4.5% ± 0.6%) were generated by PMP colonies (**Fig. 5a** and **Table 1**) and could be found in the same clonal colonies as insulin$^+$ cells. Glucagon, somatostatin and insulin defined non-overlapping differentiated cell populations.

To determine whether PMPs represent a general pancreatic precursor capable of generating exocrine cell types, we tested differentiated clonal PMP colonies for the presence of pancreatic ductal epithelial and acinar cells. Colonies were stained with CK-19, which marks ductal epithelial cells, and amylase, which marks exocrine acinar cells. Although primary ductal cells stained strongly with CK-19, no positive cells from differentiated PMPs were observed. It is possible that PMPs may generate immature ductal cells, or cells with a cytokeratin expression profile distinct from primary cells. PMPs did generate amylase$^+$ acinar cells (6.2% ± 1.2%) (**Fig. 5b** and **Table 1**), suggesting that PMPs are common precursors for both exocrine and endocrine lineages of the pancreas.

During embryonic development, neural cells arise from ectoderm and pancreatic cells arise from endoderm. Thus, single PMPs generated cell types characteristic of two different primary germ layers. To determine whether PMPs could generate other nonneural ectodermal derivatives, we tested PMP colonies for the generation of epidermal

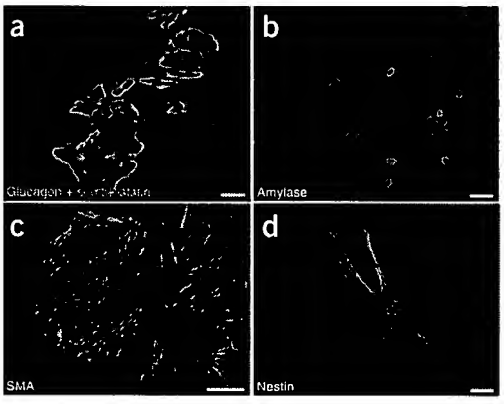


Figure 5 PMP colonies generate multiple islet endocrine subtypes and exocrine cells. (**a**) When individual PMP colonies were differentiated, they were found to generate glucagon$^+$ α-cells (green) and somatostatin$^+$ δ-cells (red). Cells coexpressing these hormones were never observed. Note that this field depicts only a portion of a larger differentiated PMP colony. The arrangement of endocrine cells in these colonies is suggestive of either multiple divisions of one local progenitor cell within the colony, or that there may be a type of 'community effect' whereby endocrine cells of similar phenotype tend to differentiate in close contact with each other. (**b**) PMP colonies generated cells characteristic of the exocrine compartment of the pancreas, amylase$^+$ acinar cells. (**c**-**d**) A large proportion of the cells generated by individual clonal PMP colonies were large, flat cells with characteristic morphology and arrangement that expressed SMA (**c**) and nestin (**d**), typical of pancreatic stellate cells. All nuclei were counterstained with DAPI (blue) for purposes of quantification. Refer to **Table 1** for relative proportions of each pancreatic cell type produced by PMPs. Scale bars, 25 μm.

Table 1 Comparison of the mean percentages of neural and pancreatic cell progeny generated from murine adult pancreas (PMP) colonies and adult forebrain-derived neurospheres (mean % ± s.e.m.)

	Neural cell types			Pancreatic cell types					
	Neurons (β3-tubulin$^+$)	Astrocytes (GFAP$^+$)	Oligo-dendrocytes (O4$^+$)	β-Cells (insulin$^+$)	α-cells (glucagon$^+$)	δ-cells (somatostatin$^+$)	Acinar Cells (amylase$^+$)	Stellate Cells (SMA$^+$)	Stellate/neural precursor cells[b] (nestin$^+$)
PMP Colony	26.4 ± 3.8[a]	7.4 ± 1.3[a]	2.4 ± 0.7	4.7 ± 1.0[a]	6.3 ± 2.0[a]	4.5 ± 0.6[a]	6.2 ± 1.2[a]	57.4 ± 7.0[a]	49.6 ± 2.9[a]
Forebrain neurosphere	3.7 ± 0.6	84.2 ± 1.4	4.3 ± 1.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	9.5 ± 1.8[b]

The table shows the fundamental differences in cell lineage potential of clonal colonies generated from the adult forebrain or adult pancreas. The numbers of each cell type are expressed as a percentage of DAPI-positive nuclei.
[a]Significant difference in the proportion of the indicated cell type generated by pancreas colonies compared to neurospheres ($P < 0.05$). PMP colonies generate a significantly higher proportion of neurons than adult brain-derived neurospheres, but significantly fewer astrocytes. Pancreatic cell types were detected only in PMP colonies and were not detected in differentiated brain-derived neurospheres. [b]Pancreatic stellate cells are known to express both SMA and nestin, and both of these markers were expressed in PMP progeny with characteristic large, flat morphology. However, because there was no SMA expression in neurosphere cells, the nestin immunoreactivity in these cultures likely represents a subpopulation of undifferentiated neural precursors. Further, nestin and β_3-tubulin are sometimes coexpressed as newly generated neurons differentiate.

cells by immunostaining with CK-1, CK-5 and CK-10. Neither islet nor ductal-derived pancreas colonies generated CK-1$^+$, CK-5$^+$ or CK-10$^+$ epidermal cells, suggesting that PMPs are restricted in the types of ectodermal derivatives that they can generate (that is, only neuroectodermal). To determine whether PMP colonies possessed a more general endodermal character, we assayed individual colonies by RT-PCR for the presence of the early endoderm markers GATA-4 and HNF3β. PMP colonies did not express these markers (**Fig. 6a**). Differentiated PMP colonies were also assayed for hepatocytes (another endodermal cell type) by immunocytochemistry for fumarylacetoacetate hydrolase (FAH); none were detected. These data suggest that PMPs are not generalized endodermal precursors.

From the preceding analyses, we could account for 50% of the differentiated progeny of individual clonal PMP colonies. The remaining unmarked cells were large and flat, with large nuclei, arranged in sheets. These cells expressed nestin (49.6% ± 2.9% of total DAPI$^+$ nuclei) and smooth muscle actin (SMA) (57.4% ± 7.0% of total DAPI$^+$ nuclei) (**Fig. 5c,d** and **Table 1**). Because nestin and SMA were expressed in a morphologically overlapping cell population, these cells likely represent pancreatic stellate cells[26]. Stellate cells are blood vessel–associated pericytes found in the pancreas and liver, which can transdifferentiate into fibroblastoid-type proliferative cells under certain conditions[26].

The self-renewal of PMPs is limited

Stem cells are defined by two properties: their multipotentiality and long-term self-renewal capacity[27]. To determine the capacity of pancreatic precursors for self-renewal, we dissociated individual clonal colonies into single cells and replated in the same mitogen-containing media used for the isolation of primary colonies, and then assayed after 7–14 d *in vitro* for the presence of secondary colonies. Only very few (<1%) of the primary PMP colonies generated small secondary colonies, suggesting that pancreatic precursors do not undergo many self-renewing divisions under these culture conditions (see also **Supplementary Notes** online).

PMPs exist in both nestin$^+$ and nestin$^-$ pancreatic cell fractions

Although PMP colonies expressed nestin according to RT-PCR analysis, this did not clarify whether colony-initiating cells are nestin$^+$. To determine whether PMPs are nestin$^+$, we used a transgenic mouse in which enhanced GFP is controlled by the nestin promoter[28]. Islet and ductal cells were analyzed for GFP expression by fluorescence-activated cell sorting (FACS), sorted into nestin$^+$ (**Supplementary Fig. 2a**, online) and nestin$^-$ fractions (**Supplementary Fig. 2b**, online) and cultured. Approximately 5% of islet fraction cells and 1% of ductal fraction cells were nestin$^+$. However, the nestin$^+$ subpopulation was not enriched for PMP colony-forming cells. Indeed, 1/4,286 nestin$^+$ cells and 1/2,514 nestin$^-$ cells formed colonies, suggesting that the nestin$^+$ cells were slightly depleted of PMP colony-forming cells. These FACS results were confirmed with a second independent nestin-GFP transgenic mouse line[29], strongly suggesting that nestin expression cannot be used to predict PMP identity.

Although colonies formed from both nestin$^+$ and nestin$^-$ cells, all of the colonies assayed at the end of the culture period were nestin$^+$

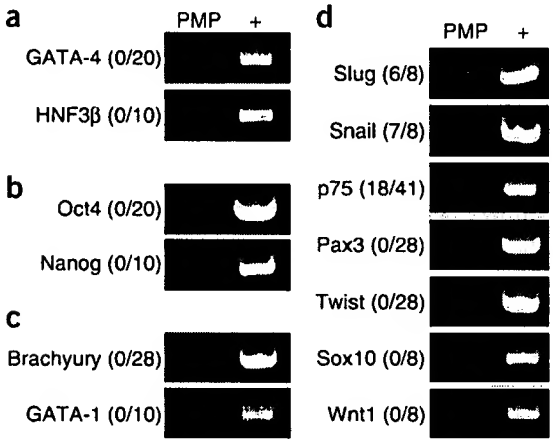


Figure 6 PMPs are not general endodermal or mesodermal precursors, nor are they ES cell–like stem cells or neural crest precursors. (**a**) Individual PMP colonies were assayed by RT-PCR for the presence of the early endoderm markers GATA-4 and HNF3β. None of the colonies tested expressed either marker, suggesting that PMPs are not generalized endodermal precursors. (**b**) mRNA for Oct4 and Nanog, proteins encoded by genes characteristic of ES cells, was not detected in any of the single clonal PMP colonies assayed, suggesting that PMPs are not ES cell–like pluripotent stem cells. (**c**) Brachyury and GATA-1, markers of mesodermal tissue, were not detected by RT-PCR in PMP colonies, suggesting that PMPs are not of mesodermal origin. (**d**) Clonal PMP colonies do not exhibit a characteristic neural crest progenitor profile. Although PMP colonies do express Slug and Snail, and a proportion of them express detectable levels of p75, they do not express many other characteristic neural crest markers including Pax3, Twist, Sox10 or Wnt1 by RT-PCR analysis. Only single colony RNA isolates that were found to express β-actin were considered. Note that positive control (+) bands appear brighter because of the greater amount of starting RNA in comparison to single PMP colonies.

(**Supplementary Fig. 2a,b** online). The transgene expression was confirmed by independent experiments to detect endogenous nestin protein by immunocytochemistry (**Supplementary Fig. 2c** online). Thus, consistent with the finding by RT-PCR analysis that PMP colonies are nestin$^+$ (**Fig. 1d**), even the PMPs contained within the nestin$^-$ fraction of cells (or their progeny within the colony) expressed nestin during proliferation and colony formation. These nestin$^+$ cells did not coexpress CD31 or E-cadherin according to immunocytochemistry analysis, suggesting that the nestin$^+$ cells present in undifferentiated PMP colonies are not endothelial or epithelial cells, respectively.

PMPs are not pluripotent embryonic stem (ES) cell–like cells

As nestin$^+$ cells were not enriched in PMPs, we investigated other candidate markers (see **Supplementary Notes** online). It has been suggested that a subpopulation of the pluripotent stem cells present in the inner cell mass of the pre-implantation embryo might never differentiate, but instead may persist and seed adult tissues. It has been hypothesized that such rare pluripotent cells may be responsible for recent observations of unexpected adult somatic tissue plasticity[30]. Oct4 is a transcription factor critical to the development of totipotent cells[31]. To determine whether Oct4 or Nanog (which is also expressed in pluripotent ES cells[32]) were transcribed in pancreatic precursors, we analyzed primary islet cells and single PMP colonies by RT-PCR for Oct4 and Nanog mRNA. Neither PMP colonies (**Fig. 6b**) nor primary islet cells expressed detectable levels of Oct4 or Nanog mRNA. These results indicate that PMPs do not correspond to a population of putative pluripotent ES cell–like stem cells in adult tissues.

PMPs are not mesodermal in origin

It has been suggested that primitive mesodermal stem cells originating from the bone marrow exist in multiple adult tissues and may adopt tissue-specific characteristics depending on the local environment[33]. Stem cell antigen 1 (Sca-1) is a cell surface protein of the *Ly6* gene family expressed by bone marrow–derived hematopoietic stem cells[34]. To determine whether PMPs were Sca-1$^+$ and thus related to primitive mesodermal stem cells, we labeled primary islet and ductal cells with a Sca-1 antibody and sorted them by FACS. Although 9% of islet cells and 15% of ductal cells were Sca-1$^+$, none of the Sca-1$^+$ cells formed pancreatic colonies. To confirm that PMPs are not mesodermal in origin, we carried out single colony RT-PCR for mesoderm markers Brachyury and GATA-1. None of the clonal colony samples tested was positive for Brachyury or GATA-1 mRNA (**Fig. 6c**). Differentiated PMP colonies were also analyzed for MyoD, a marker of mesoderm-derived myoblasts and differentiated skeletal muscle cells[35]. No MyoD$^+$ cells were found in differentiated PMP colonies. Thus, PMPs are neither Sca-1$^+$, GATA-1$^+$, nor Brachyury$^+$, and do not generate typical mesodermal progeny, suggesting that they do not represent a primitive mesodermal precursor.

PMPs are not neural crest cells

Nestin-positive precursor cells that can produce neurons *in vitro* have been isolated from adult skin (skin-derived precursors or SKPs)[36], and may represent a neural crest derivative[37]. Because PMPs are a similarly unusual source of neurons, pancreas colonies were assayed for the expression of neural crest markers by RT-PCR.

Clonal PMP colonies do not express the neural crest markers Pax3[38] or Twist[39] (**Fig. 6d**), markers expressed by SKPs[37], nor do they express neural crest markers Sox10[40] or Wnt1[41] (**Fig. 6d**). Most PMP colonies expressed Slug and Snail[42], and some expressed detectable levels of p75 neurotrophin receptor mRNA (**Fig. 6d**), which is expressed by neural crest stem cells[43]. However, p75 is also expressed in forebrain neurons[44], embryonic islets[45] and in the present study, brain-derived neurospheres (**Supplementary Table 1** online). Although Slug and Snail were detected, PMPs did not express the full cluster of markers that have been found coexpressed in neural crest stem cells or progenitors derived from neural crest[37]. These data suggest that PMPs do not exhibit a typical neural crest progenitor profile and are not neural crest derivatives.

DISCUSSION

We describe the isolation and characterization of a pancreas-derived multipotential precursor cell. PMPs are present at low frequency (~0.02%–0.03%) in both nestin$^+$ and nestin$^-$ cell fractions from both islet and ductal isolates. Single PMPs are capable of proliferation and colony formation *in vitro*, as determined by mixing experiments of marked and unmarked cells and, more definitively, by single cell analyses. PMP colonies express both neural and pancreatic precursor markers, and generate all three types of neural progeny (neurons, astrocytes and oligodendrocytes), in addition to cells with characteristics of three islet endocrine subtypes, β-cells, α-cells and δ-cells, as well as exocrine acinar cells and pancreatic stellate cells. In addition to expressing a large array of typical β-cell markers, the *de novo* generated β-like cells contain insulin and are functional as demonstrated by glucose-stimulated $[Ca^{2+}]_i$ responsiveness and insulin release. The insulin content of the PMP-generated cells was approximately one-third that of primary islet cells, in spite of the fact that islet cells contain a significantly higher proportion of β-cells than do differentiated PMP colonies. This would seem to suggest the unlikely possibility that per cell, PMP-derived cells contain more insulin than primary β-cells. Although the comparison to PMP-derived β-like cells using the same assay yields valid relative proportions, it is prudent to note that the estimated insulin content for primary β-cells in the present study is somewhat lower than that typically reported in the literature. Literature values for the insulin content of primary islets range from 667 ng insulin/μg DNA[46] to 5500 ng insulin/μg DNA[47]. Calculations based on these literature values suggest that PMP-derived β-like cells contain somewhere between 11.6% and 96% of the insulin level of primary β-cells. It could be that the PMP-derived cells are less efficient at releasing stored insulin and thus only appear to have a greater insulin production capacity than primary β-cells. It is formally possible that other, heretofore unrecognized differences exist between primary β-cells and PMP-generated β-cells, which therefore may be more appropriately termed "β-like" cells.

The capacity of PMPs to generate neural and pancreatic progeny may be explained by two alternative hypotheses: (i) the pancreas and brain may have a common embryological origin from an ectodermal/endodermal precursor population that exists during early embryonic development; or (ii) the similarity in gene expression patterns of the brain and pancreas (e.g., nestin, Ngn3, Beta2/NeuroD, Pax6) indicate that evolution has reused the same 'toolbox' of genes in two otherwise unrelated tissues.

There is little direct evidence to support the notion that an ectodermal/endodermal bipotential precursor exists during embryonic development. A recent study isolated an ES cell derivative that, when exposed to early inductive signals, may generate insulin$^+$ cells and immature neurons[48]. However, doubt has been cast on ES cell–derived insulin$^+$ cells due to lack of PDX-1 and C-peptide expression, the demonstration that insulin is not released from these cells in a glucose-dependent manner and the fact that these cells form teratomas *in vivo*[49]. Nonetheless, it is interesting to speculate that perhaps

a population of ectodermal/endodermal precursors does exist transiently *in vivo* during development, and that PMPs may represent a small subpopulation of these precursors that persist in the adult pancreas, still capable of generating both β-like cells and neural cell types. It is notable that neuronal cell bodies lie in close juxtaposition to islet β-cells in the postnatal pancreas[50] and may play a role in the coordination of insulin release. This relationship is intriguingly suggestive of the need for a bipotential ectodermal/endodermal precursor cell to persist. Alternatively, the similarity in developmental gene expression program or 'toolbox' may permit pancreatic precursor cells to generate neurons when they receive the appropriate signals, as in our neurosphere culture system. The present data are not able to discriminate between these two possibilities.

The criteria for defining a precursor cell as a stem or progenitor have been rigorously defined[27]. Because of their apparently limited self-renewal capacity, PMPs may be most appropriately termed progenitor cells, and indeed there are other examples of adult tissues seeded with relatively restricted progenitor cells[8]. However, it remains possible that the appropriate culture conditions have not yet been determined for their robust self-renewal. Indeed, although hematopoietic stem cells have been studied for decades and have been demonstrated to self-renew according to stringent *in vivo* assays[51], conditions for the self-renewal in culture have only been recently described[52].

There have been a number of other striking studies suggestive of the multi-germ layer lineage potential of adult bone marrow cells[53], neural stem cells[54] and perinatal inner ear cells[12], but to date such cells have not been isolated from the adult pancreas. PMPs represent the first clonally characterized adult somatic cell type from the pancreas capable of reliably and reproducibly generating progeny characteristic of more than one embryonic primary germ layer.

The role of PMPs *in vivo* will be the subject of future study. Recently it has been suggested that differentiated β-cells, rather than putative pancreatic precursors, may represent the most robust source of new β-cells *in vivo*[55]. However, this study used insulin expression to mark β-cells, a technique that also may mark putative precursor cells (including PMPs) that may express insulin *in vivo*. It has also been suggested that transformation events occur *in vitro* and might cause the generation of unexpected cell types from various somatic precursor cells. However, transformation events manifest themselves over longer culture periods than those used in our study, and have different phenotypes in different cell isolates[56]. For the consistently observed PMP-derived neurons and pancreatic endocrine cells to be the result of a transformation, the identical transformation event would have had to occur in each of the more than 100 clonal colonies assayed from more than 14 separate experiments. This suggests that the unusual combination of cell types generated by PMPs was not the result of transformation events.

There is debate regarding the identity of the nestin$^+$ adult pancreas cells[3]. Because PMPs were present in nestin$^+$ and nestin$^-$ cell fractions, PMP identity cannot be predicted by nestin expression. Other studies have similarly suggested that nestin expression is not related to pancreatic precursor identity[57,58,59]. However, PMP colonies derived from nestin$^+$ and nestin$^-$ precursor cells ultimately expressed nestin in the majority of cells, suggesting that nestin may be expressed by progenitors downstream of colony-initiating PMPs. Epithelial cells were not generated by PMPs, nor did PMP-derived nestin$^+$ cells coexpress the endothelial cell marker CD31 or the epithelial marker E-cadherin, indicating that they may be a different nestin$^+$ progenitor cell than those suggested to represent rare pancreatic epithelial cell progenitors *in vivo*[60,61,62,63]. Moreover, in differentiated PMP colonies, nestin expression was associated with pancreatic stellate (mesenchymal) cells, as has been previously described[26,64].

We investigated the relationship between PMPs and other previously described adult precursors. PMPs do not express Oct4 or Nanog, markers expressed by ES cells. Thus, PMPs are likely not pluripotent cells that escaped differentiation and seeded the adult pancreas, a suggestion that has been made by others[30] to explain recent reports of the unexpected plasticity of adult precursors. Oct4 also is expressed by multipotential adult precursor cells (MAPCs) recently demonstrated to arise from long-term cultures of marrow stroma and to generate progeny of all three germ layers[53]. Because PMPs do not express Oct4 or have mesodermal character, they are unlikely to represent a novel source of MAPCs or other bone marrow–derived stem cells of mesodermal origin[33,65]. Neural crest markers are not expressed by PMPs, suggesting they are not derived from this tissue. Thus, we posit that PMPs represent a novel intrinsic adult pancreatic precursor cell. No pancreatic cells have previously been identified at the single-cell level as being capable of generating multiple pancreatic and neural cell types. As such, PMPs may represent a promising source of cells for replacement therapies.

METHODS

Animals, cell isolation and culture. The mice used in these studies included 6-week-old male Oct4-EGFP animals that express enhanced GFP under the control of the Oct4 promoter (a kind gift from A. Nagy), nestin-EGFP animals that express enhanced GFP under the control of the 5′ nestin promoter and nestin second-intron enhancer[28], GFP animals that constitutively express GFP in all cells (Jackson) and wild-type BalbC animals (Charles River). Islets were isolated by collagenase digestion of the pancreas and Ficoll density gradient centrifugation. After centrifugation islets were handpicked for further purification[66]. Ductal tissue was similarly handpicked to ensure purity.

Isolated islets and ductal tissue were then incubated with trypsin (Sigma) at 37 °C and triturated with a small-borehole siliconized pipette into a single cell suspension. Viable cells were counted using Trypan Blue (Sigma) exclusion and plated at 20 cells μl^{-1} or less in defined serum-free medium (SFM)[67] containing 1x B27 (Gibco-BRL), 10 ng ml^{-1} FGF2 (Sigma), 2 $\mu g\ ml^{-1}$ heparin (Sigma) and 20 ng ml^{-1} EGF (Sigma) for 7–14 d *in vitro*. For some experiments, the following growth factors were added: 100 pM hepatocyte growth factor (Sigma), 10 ng ml^{-1} keratinocyte growth factor (Calbiochem), 10 ng ml^{-1} insulin-like growth factor-1 (Upstate Biotech), 2 nmol L^{-1} Activin-A (Sigma), 10 mM nicotinamide (Sigma) and 10 nM exendin-4 (Sigma).

For clonal analysis, primary cells were diluted to a density of 0.05 cells μl^{-1} and plated in Nunclon 96-well plates (Nalge Nunc International). Each well was scored after plating for the presence of a single cell. Only wells that contained single cells at the outset of the culture period were subsequently assayed for colony formation.

For differentiation, whole individual pancreas colonies were removed from the aforementioned mitogen-containing media and transferred to wells coated with Matrigel basement membrane matrix (15.1 mg ml^{-1} stock diluted 1:25 in SFM, Becton-Dickinson) in SFM containing 1% FBS without dissociation. As the colony differentiates, cells migrate out of the spherical colony to form a flat monolayer. To ensure accurate assay of the progeny from single pancreatic precursors, each well contained only a single clonal pancreas colony. Neurospheres were generated from adult mice for comparison purposes as described previously[8].

FACS analysis. Islet and ductal cells were isolated as described, and cells were sorted with an EPICS Elite Cell Sorter (Beckman-Coulter). In the case of nestin-EGFP and Oct4-EGFP transgenic cells, separate single cell suspensions of islet and ductal cells were sorted into separate fractions based on EGFP fluorescence. For the Sca-1 sorting experiment, cells were first labeled with PE-Sca-1 mouse monoclonal antibody (1:250; Pharmingen) and sorted into separate cell fractions based on PE (phyco-erythrin) fluorescence.

Immunocytochemistry, cell quantification and statistical analysis. Fixation and immunocytochemical analysis of pancreas colonies were done as described previously for neurospheres[8]. See **Supplementary Methods** online for a list of the primary and secondary antibodies used, as well as positive control tissues for each antibody. For cell quantification, the numbers of neurons, astrocytes, oligodendrocytes, β-cells, α-cells, δ-cells, acinar cells, stellate cells, stellate/neural precursor cells were determined by counting the numbers of β_3-tubulin$^+$, GFAP$^+$, O4$^+$, insulin$^+$ cells, glucagon$^+$, somatostatin$^+$, amylase$^+$, SMA$^+$ and nestin$^+$ cells respectively, as a percentage of DAPI$^+$ nuclei in at least three photographed fields of differentiated cells per colony ($n \geq 10$ colonies). The absolute number of cells counted per cell type to determine the percentages (**Table 1**) ranged between 2,000 to 4,000 cells each. Statistical analyses consisted of Student's *t*-tests. A P value <0.05 was considered to represent a significant difference between groups.

RT-PCR analysis. Total RNA was extracted from individual colonies using an RNeasy extraction kit (Qiagen). Reverse transcription and PCR were carried out using a OneStep RT-PCR kit (Qiagen) in a GeneAmp PCR System 9700 (Applied Biosystems) according to kit instructions. PCR reactions were performed for 35–40 cycles because of the relatively small amount of starting material involved in single-colony RT-PCR. It is important to note that it is difficult to draw conclusions about mRNA quantity from these methods. All samples were treated with DNAse to avoid contamination with genomic DNA. Controls run without reverse transcriptase did not produce bands. Forward and reverse primers (5′–3′), expected product size, annealing temperatures and positive control tissues can be found in the **Supplementary Methods** online. Only single colony RNA isolates that were found to express β-actin were considered for further analysis. If β-actin was found in a single colony RNA isolate but the gene of interest was not, five colonies were pooled and reassayed. When expression was found in pooled but not single samples, this result was interpreted as mRNA presence in PMP colonies, but perhaps at low levels.

RIP-YFP adenovirus and $[Ca^{2+}]_i$ imaging studies. An adenovirus in which the expression of enhanced yellow fluorescent protein (EYFP) was placed under the control of the rat insulin II gene promoter (RIP2) (AdRIP2EYFP) was constructed as described[22]. Expression of EYFP has been demonstrated to be restricted to infected insulin$^+$ β-cells in whole islets of Langerhans[22]. PMP colonies were infected with AdRIP2EYFP for 48 h from day 5 to day 7 of differentiation. Colonies were trypsinized, dissociated and replated on laminin/polyornithine-coated glass coverslips for 24 h in RPMI-1640 medium containing 5 mM glucose, 10% FCS and 10 mM HEPES before imaging. Experiments were done in a KRB solution consisting of (in mM): 129 NaCl, 4.8 KCl, 5 $NaHCO_3$, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 10 HEPES and 0.1% BSA. Individual RIP-YFP$^+$ cells were visualized and Ca^{2+} imaging using Fura2 was done on these single cells as previously described[68].

Insulin release and content studies. PMP colonies were pooled and differentiated (8 per well, 96-well Matrigel-coated plates) for 7 d as described. Twenty-four hours before secretion studies, the medium was changed to supplemented RPMI-1640 medium as outlined above. Differentiated PMP colonies were preincubated in low glucose (2.5 mM) KRB solution (LG-KRB) for 1 h. The solution was changed to 150 μl of fresh LG-KRB and the cultures were incubated for 1.5 h to establish the basal level of insulin release. Cultures were incubated for a further 1.5 h in either LG-KRB alone or with experimental agents (20 mM glucose, 30 nM GLP-1, 10 mM TEA, 100 μM verapamil or 100 μM carbachol). Insulin was measured using an RIA kit (Linco) (detection range, 0.1 – 10 ng/ml). Insulin release during the experimental 1.5 h incubation was compared to the level determined during the basal 1.5-h incubation period for each individual well to obtain a percent change. The data are expressed relative to the percent change measured for the LG-KRB to the LG-KRB alone condition.

Differentiated PMP culture samples collected for insulin content determination were prepared as described above for insulin release. Primary islets (10 islets/sample) were isolated as described above and cultured for 24 hours in Ham's F-10 medium prior to insulin content determination. Insulin content samples ($n = 8$ samples from 3 separate experiments) were incubated overnight at 4°C in acid/ethanol to extract total insulin and DNA. Insulin was measured using RIA, and DNA was quantified spectrophotometrically using a BioPhotometer 6131 (Eppendorf).

Note: Supplementary information is available on the Nature Biotechnology website.

ACKNOWLEDGMENTS

We thank D. Dixon for excellent tissue isolation and purification, C. Smith for expert assistance with FACS analysis, A. Nagy for the gift of Oct4-GFP transgenic mice, M. Yamaguchi for the gift of nestin-GFP transgenic mice, M. Grompe for the gift of anti-FAH chicken polyclonal antibody and R.D.G. McKay for the gift of anti-nestin rabbit polyclonal antibody. This study was supported by the Canadian Stem Cell Network and the Canadian Institutes of Health Research.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 19 December 2003; accepted 7 June 2004
Published online at http://www.nature.com/naturebiotechnology/

1. Lechner, A. & Habener, J.F. Stem/progenitor cells from adult tissues: potential for the treatment of diabetes mellitus. *Am. J. Physiol. Endocrinol. Metab.* **284**, E259–E266 (2003).
2. Ramiya, V.K. *et al.* Reversal of insulin-dependent diabetes using islets generated *in vitro* from pancreatic stem cells. *Nat. Med.* **6**, 278–282 (2000).
3. Hunziker, E. & Stein, M. Nestin-expressing cells in the pancreatic islets of Langerhans. *Biochem. Biophys. Res. Commun.* **271**, 116–119 (2000).
4. Zulewski, H. *et al.* Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. *Diabetes* **50**, 521–533 (2001).
5. Abraham, E.J., Leech, C.A., Lin, J.C., Zulewski, H. & Habener, J.F. Insulinotropic hormone glucagon-like peptide-1 differentiation of human pancreatic islet-derived progenitor cells into insulin-producing cells. *Endocrinology* **143**, 3152–3161 (2002).
6. Lendahl, U., Zimmerman, L.B. & McKay, R.D. CNS stem cells express a new class of intermediate filament protein. *Cell* **60**, 585–595 (1990).
7. Weiss, S. *et al.* Is there a neural stem cell in the mammalian forebrain? *Trends Neurosci.* **19**, 387–393 (1996).
8. Seaberg, R.M. & van der Kooy, D. Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. *J. Neurosci.* **22**, 1784–1793 (2002).
9. Hadjantonakis, A.-K., Gertsenstein, M., Ikawa, M., Okabe, M. & Nagy, A. Generating green fluorescent mice by germline transmission of green fluorescent ES cells. *Mech. Dev.* **76**, 79–90 (1998).
10. Morshead, C.M., Garcia, A.D., Sofroniew, M.V. & van der Kooy, D. The ablation of glial fibrillary acidic protein-positive cells from the adult central nervous system results in the loss of forebrain neural stem cells but not retinal stem cells. *Eur. J. Neurosci.* **18**, 76–84 (2003).
11. Tropepe, V. *et al.* Retinal stem cells in the adult mammalian eye. *Science* **287**, 2032–2036 (2000).
12. Li, H., Liu, H. & Heller, S. Pluripotent stem cells from the adult mouse inner ear. *Nat. Med.* **9**, 1293–1299 (2003).
13. Jonsson, J., Carlsson, L., Edlund, T. & Edlund, H. Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* **371**, 606–609 (1994).
14. Guz, Y. *et al.* Expression of murine STF-1, a putative insulin gene transcription factor, in beta cells of pancreas, duodenal epithelium and pancreatic exocrine and endocrine progenitors during ontogeny. *Development* **121**, 11–18 (1995).
15. Kritzik, M.R. *et al.* PDX-1 and Msx-2 expression in the regenerating and developing pancreas. *J. Endocrinol.* **163**, 523–530 (1999).
16. Zhang, Y.-Q. & Sarvetnick, N. Development of cell markers for the identification and expansion of islet progenitor cells. *Diabetes Metab. Res. Rev.* **19**, 363–374 (2003).
17. Sommer, L., Ma, Q. & Anderson, D.J. Neurogenins, a novel family of atonal-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. *Mol. Cell. Neurosci.* **8**, 221–241 (1996).
18. Bylund, M., Andersson, E., Novitch, B.G. & Muhr, J. Vertebrate neurogenesis is counteracted by Sox1–3 activity. *Nat. Neurosci.* **6**, 1162–1168 (2003).
19. Guillemot, F. & Joyner, A.L. Dynamic expression of the murine achaete-scute homologue Mash-1 in the developing nervous system. *Mech. Dev.* **42**, 171–185 (1993).
20. Liu, Y. & Rao, M. Oligodendrocytes, GRPs and MNOPs. *Trends Neurosci.* **26**, 410–412 (2003).
21. Rajagopal, J., Anderson, W.J., Kume, S., Martinez, O.I. & Melton, D.A. Insulin staining of ES cell progeny from insulin uptake. *Science* **299**, 363 (2003).
22. Kang, G. *et al.* Epac-selective camp analog 8-pCPT-2′-*O*-Me-cAMP as a stimulus for Ca^{2+}-induced Ca^{2+} release and exoctyosis in pancreatic β-cells. *J. Biol. Chem.* **278**, 8279–8285 (2003).
23. MacDonald, P.E. *et al.* The multiple actions of GLP-1 on the process of glucose-stimulated insulin secretion. *Diabetes* **51**, S434–S442 (2002).

24. Roe, M.W. *et al.* Expression and function of pancreatic beta-cell delayed rectifier K^+ channels. Role in stimulus-secretion coupling. *J. Biol. Chem.* **271**, 32241–32246 (1996).
25. Ahren, B., Karlsson, S. & Lindskog, S. Cholinergic regulation of the endocrine pancreas. *Prog. Brain Res.* **84**, 209–218 (1990).
26. Lardon, J., Rooman, I. & Bouwens, L. Nestin expression in pancreatic stellate cells and angiogenic endothelial cells. *Histochem. Cell Biol.* **117**, 535–540 (2002).
27. Seaberg, R.M. & van der Kooy, D. Stem and progenitor cells: the premature desertion of rigorous definitions. *Trends Neurosci.* **26**, 125–131 (2003).
28. Mignone, J., Kukekov, V., Chiang, A.S., Steindler, D. & Enikolopov, G. Neural stem and progenitor cells in nestin-GFP transgenic mice. *J. Comp. Neurol.* **469**, 311–324 (2004).
29. Sawamoto, K. *et al.* Generation of dopaminergic neurons in the adult brain from mesencephalic precursor cells labeled with a nestin-GFP transgene. *J. Neurosci.* **21**, 3895–3903 (2001).
30. Weissman, I.L. Stem cells: Units of development, units of regeneration, and units in evolution. *Cell* **100**, 157–168 (2000).
31. Nichols, J. *et al.* Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95**, 379–391 (1998).
32. Chambers, I. *et al.* Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113**, 643–655 (2003).
33. Gussoni, E. *et al.* Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* **401**, 390–394 (1999).
34. Spangrude, G.J., Heimfeld, S. & Weissman, I.L. Purification and characterization of mouse hematopoietic stem cells. *Science* **241**, 58–62 (1988).
35. Eftimie, R., Brenner, H. & Buonanno, A. Myogenin and MyoD join a family of skeletal muscle genes regulated by electrical activity. *Proc. Natl. Acad. Sci. USA* **88**, 1349–1353 (1991).
36. Toma, J.G. *et al.* Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat. Cell Biol.* **3**, 778–784 (2001).
37. McKenzie, I. *et al.* Evidence that skin-derived precursor cells (skps) are an endogenous neural crest-derived precursor. *Abstr. Soc. Neurosci.* **28**, 9 (2003).
38. Goulding, M.D., Chalepakis, G., Deutsch, U., Erselius, J.R. & Gruss, P. Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. *EMBO J.* **10**, 1135–1147 (1991).
39. Stoetzel, C., Weber, B., Bourgeois, P., Bolcato Bellemin, A.L. & Perrin Schmitt, F. Dorso-ventral and rostro-caudal sequential expression of M-twist in the postimplantation murine embryo. *Mech. Dev.* **51**, 251–263 (1995).
40. Honore, S.M., Aybar, M.J. & Mayor, R. Sox10 is required for the early development of the prospective neural crest in *Xenopus* embryos. *Dev. Biol.* **260**, 79–96 (2003).
41. Lee, H.Y. *et al.* Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells. *Science* **303**, 1020–1023 (2004).
42. Aybar, M.J., Nieto, M.A. & Mayor, R. Snail precedes slug in the genetic cascade required for the specification and migration of the *Xenopus* neural crest. *Development* **130**, 483–494 (2003).
43. Morrison, S.J., White, P.M., Zock, C. & Anderson, D.J. Prospective identification, isolation by flow cytometry, and *in vivo* self-renewal of multipotent mammalian neural crest stem cells. *Cell* **96**, 737–749 (1999).
44. Peterson, D.A., Leppert, J.T., Lee, K.-F. & Gage, F.H. Basal forebrain neuronal loss in mice lacking neurotrophin receptor p75. *Science* **277**, 837–838 (1997).
45. Scharfmann, R. Neurotrophin and neurotrophin receptors in islet cells. *Horm. Metab. Res.* **29**, 294–296 (1997).
46. Welsh, M. *et al.* The FRK/RAK tyrosine kinase participates in cytokine-induced islet cell toxicity. *Biochem J.* published online; doi: 10.1042/BJ20040285 (2004).
47. Garcia-Ocana, A. *et al.* Transgenic overexpression of hepatocyte growth factor in the beta-cell markedly improves islet function and islet transplant outcomes in mice. *Diabetes* **50**, 2752–2762 (2001).
48. Lumelsky, N. *et al.* Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* **292**, 1389–1394 (2001).
49. Sipione, S., Eshpeter, A., Lyon, J.G., Korbutt, G.S. & Bleackley, R.C. Insulin expressing cells from differentiated embryonic stem cells are not beta cells. *Diabetologia* **47**, 499–508 (2004).
50. Persson-Sjogren, S., Zashihin, A. & Forsgren, S. Nerve cells associated with the endocrine pancreas in young mice: An ultrastructural analysis of the neuroinsular complex type I. *Histochem. J.* **33**, 373–378 (2001).
51. Benveniste, P., Cantin, C., Hyam, D. & Iscove, N.N. Hematopoietic stem cells engraft in mice with absolute efficiency. *Nat. Immunol.* **4**, 708–713 (2003).
52. Antonchuk, J., Sauvageau, G. & Humphries, R.K. HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. *Cell* **109**, 39–45 (2002).
53. Jiang, Y. *et al.* Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**, 41–49 (2002).
54. Clarke, D.L. *et al.* Generalized potential of adult neural stem cells. *Science* **288**, 1660–1663 (2000).
55. Dor, Y., Brown, J., Martinez, O.I. & Melton, D. Adult pancreatic β-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* **429**, 41–46 (2004).
56. Morshead, C.M., Benveniste, P., Iscove, N.N. & van der Kooy, D. Hematopoietic competence is a rare property of neural stem cells that may depend on genetic and epigenetic alterations. *Nat. Med.* **8**, 268–273 (2002).
57. Piper, K. *et al.* Beta-cell differentiation during human development does not rely on nestin-positive precursors: implications for stem cell-derived replacement therapy. *Diabetologia* **45**, 1045–1047 (2002).
58. Humphrey, R.K. *et al.* Characterization and isolation of promoter-defined nestin-positive cells from the human fetal pancreas. *Diabetes* **52**, 2519–2525 (2003).
59. Treutelaar, M.K. *et al.* Nestin-lineage cells contribute to the microvasculature but not endocrine cells of the islet. *Diabetes* **52**, 2503–2512 (2003).
60. Delacour, A., Nepote, V., Trumpp, A. & Herrera, P.L. Nestin expression in pancreatic exocrine cell lineages. *Mech. Dev.* **121**, 3–14 (2004).
61. Esni, F., Stoffers, D.A., Takeuchi, T. & Leach, S.D. Origin of exocrine pancreatic cells from nestin-positive precursors in the developing mouse pancreas. *Mech. Dev.* **121**, 15–25 (2004).
62. Street, C.N. *et al.* Heterogenous expression of nestin in human pancreatic tissue precludes its use as an islet precursor marker. *J. Endocrinol.* **180**, 213–225 (2004).
63. Klein, T. *et al.* Nestin is expressed in vascular endothelial cells in the adult human pancreas. *J. Histochem. Cytochem.* **51**, 697–706 (2003).
64. Selander, L. & Edlund, H. Nestin is expressed in mesenchymal and not epithelial cells of the developing mouse pancreas. *Mech. Dev.* **113**, 189–192 (2002).
65. Polesskaya, A., Seale, P. & Rudnicki, M.A. Wnt signaling induces the myogenic specification of resident CD45+ adult stem cells during muscle regeneration. *Cell* **113**, 841–852 (2003).
66. Gotoh, M., Maki, J., Kiyoizumi, T., Satomi, S. & Monaco, A.P. An improved method for isolation of mouse pancreatic islets. *Transplantation* **40**, 437–438 (1985).
67. Tropepe, V. *et al.* Distinct neural stem cells proliferate in response to EGF and FGF2 in the developing mouse telencephalon. *Dev. Biol.* **208**, 166–188 (1999).
68. Smukler, S.R., Tang, L., Wheeler, M.B. & Salapatek, A.M.F. Exogenous nitric oxide and endogenous glucose-stimulated β-cell nitric oxide augment insulin release. *Diabetes* **51**, 3450–3460 (2002).

Prospective Isolation of Multipotent Pancreatic Progenitors Using Flow-Cytometric Cell Sorting

Atsushi Suzuki,[1,2] Hiromitsu Nakauchi,[3] and Hideki Taniguchi[2,4,5]

During pancreatic development, neogenesis, and regeneration, stem cells might act as a central player to generate endocrine, acinar, and duct cells. Although these cells are well known as pancreatic stem cells (PSCs), indisputable proof of their existence has not been reported. Identification of phenotypic markers for PSCs leads to their prospective isolation and precise characterization to clear whether stem cells exist in the pancreas. By combining flow cytometry and clonal analysis, we show here that a possible pancreatic stem or progenitor cell candidate that resides in the developing and adult mouse pancreas expresses the receptor for the hepatocyte growth factor (HGF) c-Met, but does not express hematopoietic and vascular endothelial antigens such as CD45, TER119, c-Kit, and Flk-1. These cells formed clonal colonies in vitro and differentiated into multiple pancreatic lineage cells from single cells. Some of them could largely expand with self-renewing cell divisions in culture, and, following cell transplantation, they differentiated into pancreatic endocrine and acinar cells in vivo. Furthermore, they produced cells expressing multiple markers of nonpancreatic organs including liver, stomach, and intestine in vitro. Our data strongly suggest that c-Met/HGF signaling plays an important role in stem/progenitor cell function in both developing and adult pancreas. By using this antigen, PSCs could be isolated prospectively, enabling a detailed investigation of stem cell markers and application toward regenerative therapies for diabetes. *Diabetes* 53:2143–2152, 2004

From the [1]Gene Expression Laboratories, The Salk Institute for Biological Studies, La Jolla, California; the [2]Department of Surgery, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan; the [3]Laboratory of Stem Cell Therapy, Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan; the [4]Department of Regenerative Medicine, Faculty of Medicine, Yokohama City University, Yokohama, Kanagawa, Japan; and the [5]Research Unit for Organ Regeneration, Center for Developmental Biology, The Institute of Physical and Chemical Research (RIKEN), Kobe, Hyogo, Japan.

Address correspondence and reprint requests to Hideki Taniguchi, MD, PhD, Department of Regenerative Medicine, Faculty of Medicine, Yokohama City University, 3-9 Fuku-ura, Kanazawa-ku, Yokohama, Kanagawa 236-0004, Japan. E-mail: rtanigu@med.yokohama-cu.ac.jp.

Received for publication 16 April 2003 and accepted in revised form 5 May 2004.

CAPC, cell aggregate–producing cell; EC, epithelial-like colony; ECFC, EC-forming cell; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; GLP, glucagon-like peptide; H-CFU-C, hepatic colony-forming unit in culture; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; HS, hormone sensitive; mAb, monoclonal antibody; Pdx, pancreas duodenal homeobox; PP, pancreatic polypeptide; PSC, pancreatic stem cell.

The pancreas is an organ consisting of three major different structures: the islets of Langerhans, acinar tissue, and ductal epithelia. The islets are composed of neatly arranged endocrine cell populations (glucagon-producing α-cells, insulin-producing β-cells, pancreatic polypeptide [PP]-producing γ-cells, and somatostatin-producing δ-cells). The acinar cells that secrete various enzymes, such as amylase and lipase, into the intestine, comprise a system of terminal or intercalary acini joined by ducts. During pancreatic organogenesis, these endocrine and acinar tissues seem to be developed from a common cell component associated with the pancreatic ductal epithelium (1–4). Thus, pancreatic stem cells (PSCs) responsible for both endocrine and acinar tissue formation are thought to reside in the pancreatic ducts. Identification and isolation of PSCs have generated much interest due not only to their putative developmental importance but also to their therapeutic potential.

Candidate PSC in mouse and human has been reported (5–7). They were derived from pancreatic ductal cell components and maintained in long-term culture, where they could differentiate into multilineage cell types. These cells possess characteristics very similar to those of neural (8,9), epidermal (10,11), mesenchymal (12), myogenous (13), and retinal (14) stem cells that can also propagate in culture. The value of stem cells expanded in vitro is expected to be great not only in conventional studies of their differentiation or self-renewing potential but also in therapy, such as with virus-mediated gene transfer, or as a theoretically unlimited source of cells. Characterization of those PSC candidates, however, has always been carried out retrospectively, after expansion of crudely isolated cells in culture for a relatively long period. Therefore, it has still not been determined which cells possess stem cell activity in vitro as well as in vivo. To distinguish PSCs from other cell types precisely, their prospective identification and single cell–based analysis are required.

The hematopoietic stem cells, probably the best-characterized stem cell population, were prospectively identified and isolated based on expression of cell surface antigens by flow cytometry (15–17). Although their self-renewing ability could not be maintained easily in vitro, prospective identification facilitated rapid progress toward an understanding of these cells' properties and yielded information on genes specifically expressed in this cell population (18,19). Several recent studies using fluorescence-activated cell sorting (FACS) have been conducted to isolate stem cell populations in neural tissue (20,21) and liver (22–24). These prospective studies have not only given us

information about specific characteristics of stem cells, but have also allowed us to separate them selectively from differentiated cells.

In the study reported here, we combined monoclonal antibodies and FACS to fractionate cells derived from neonatal and adult mouse pancreas based on surface marker expression. By using an in vitro clonal colony-forming assay system that was established previously (25), we analyzed cells sorted from distinct fractions and attempted to identify pancreatic stem/progenitor cells prospectively to determine their capacity for differentiation and proliferation. In order to enrich the yield of colony-forming cells and thereby permit clonal analysis of this cell class, we attempted in the present study to sort for cells expressing c-Met, the hepatocyte growth factor (HGF) receptor. Interaction between c-Met and HGF, which is mediated by a signal exchange between epithelial and mesenchymal cells (26), plays an essential part in pancreatic development (27,28). This interaction also is active in the regeneration and carcinogenesis of this organ (29–31). In addition, the number of islet-like structures budding out from cultured ductal epithelial cells is increased by HGF (6). These findings, taken together, suggest that c-Met/HGF interaction is critically responsible for growth and differentiation of pancreatic stem and progenitor cells during development, homeostatic cell turnover, and regeneration.

Our current data demonstrate that clonal colonies derived from c-Met–positive cells contained cells expressing several markers for endocrine, acinar, and ductal lineage cells. These results strongly suggest that cells initiating colony formation are defined as PSCs or common progenitors for those cell types. Several c-Met–positive sorted cells continued growing with self-renewing cell divisions, and, only from this cell subpopulation, many cell aggregates budded from monolayer cells emerged in long-term culture. These cell aggregate–producing cells (CAPCs) could differentiate clonally into multiple pancreatic lineage cells in vitro and in vivo. Furthermore, they could generate daughter cells expressing several marker genes for other organs of endodermal origin such as the liver, stomach, and intestine in vitro. These findings indicate that candidate PSCs or progenitors express c-Met, that their numbers can be enriched, and that they can be isolated using flow cytometry.

RESEARCH DESIGN AND METHODS

Dissociation of pancreatic cells. Single-cell suspensions of pancreas-constituting cells were prepared from C57BL/6 mouse neonates (1 day after birth) and adults (12 weeks old) (Clea, Tokyo, Japan). The pancreas was carefully dissected under the microscope, minced by a razor blade, and placed in Ca^{2+}-free Hanks' balanced salt solution (HBSS; Life Technologies, Gaithersburg, MD) containing 5 mmol/l $CaCl_2$ (pH 7.4) and 0.05% or 0.15% collagenase (cat no. C-5138; Sigma Chemical, St. Louis, MO) for either neonatal or adult pancreas. Digestion was carried out by gentle pipetting after incubation for 10–15 min at 37°C. Triturated pancreatic cells were washed three times in the medium before the incubation with antibodies. Cell viability exceeded 85% (neonates) or 70% (adult) as assessed by trypan blue dye exclusion.

Flow cytometry. Dissociated pancreatic cells were incubated on ice for 30 min with biotinylated anti-CD45, TER119, and c-Kit (for adult pancreas) monoclonal antibodies (mAbs) (Pharmingen, San Jose, CA) and c-Met mAb (Upstate Biotechnology, Lake Placid, NY). After three washings with staining medium (PBS containing 3% fetal bovine serum), cells were incubated with fluorescein isothiocyanate–conjugated anti-mouse IgG_{2a} mAb (Pharmingen), allophycocyanin-conjugated anti–c-Kit mAb (Pharmingen) (for neonatal pancreas), phycoerythrin-conjugated anti–Flk-1 mAb (Pharmingen), and streptavidin-labeled Texas Red (Life Technologies) on ice for 30 min. Finally, cells were washed three times and resuspended in staining medium containing propidium iodide (5 μg/ml). The labeled cells were analyzed and separated using FACS Vantage (Becton Dickinson, San Jose, CA). Establishment of the gate was based on the staining profiles of the negative control.

Cell sorting. After $CD45^+$ or $TER119^+$ hematopoietic cells from neonatal pancreas were gated out, sorting gates were set for c-Met^+ c-Kit^-, c-Met^- c-Kit^+, c-Met^+ c-Kit^+, and c-Met^- c-Kit^- subpopulations. Furthermore, by using antibodies against Flk-1, c-Met^+ c-Kit^- $CD45^-$ $TER119^-$ cells were further fractionated. Similarly, cells positive for CD45, TER119, and/or c-Kit in adult pancreas were gated out. After exclusion of $Flk\text{-}1^+$ cells from the c-Kit^- $CD45^-$ $TER119^-$ cell subpopulation, sorting gates were set for c-Met^+ $Flk\text{-}1^-$ c-Kit^- $CD45^-$ $TER119^-$ and c-Met^+ $Flk\text{-}1^+$ c-Kit^- $CD45^-$ $TER119^-$ cell subpopulations. For low-density culture analysis, sorted cells were plated on 35-mm tissue culture dishes (Becton Dickinson) at a density of 200 cells/cm^2. For single-cell culture analysis, sorted cells were plated on 96-well tissue culture plates (Becton Dickinson). The nozzle size of the FACS Vantage is 70 μm. Sort speed is <1,000 cells/s. Residual erythrocytes, debris, doublets, and dead cells were excluded by forward scatter, side scatter, and propidium iodide gating. Viability of sorted cells exceeded 90% as assessed by trypan blue exclusion.

In vitro colony assay. Sorted cells from neonatal pancreas were cultured in our fresh standard medium (23). Our standard culture medium is a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 (Sigma) with 10% fetal bovine serum (Serologicals, Norcross, GA), γ-insulin (1 mg/ml; Wako, Tokyo, Japan), dexamethasone (1×10^{-7} mol/l; Sigma), nicotinamide (10 mmol/l; Sigma), L-glutamine (2 mmol/l; Life Technologies), β-mercaptoethanol (50 mmol/l; Sigma), HEPES (5 mmol/l; Wako), and penicillin/streptomycin (Life Technologies). For single-cell culture analysis of adult pancreatic cells, we used 50% standard medium supplemented with medium conditioned by 7-day culture with STO cells. Human recombinant HGF (50 ng/ml; Sigma) and epidermal growth factor (EGF) (20 ng/ml; Sigma) were added 24 h after culture initiation. EGF was used in combination with HGF because several reports have represented a mitogenic effect of EGF for pancreatic cells (32–34). In this series of experiments, we routinely added both of them into the culture medium. To induce differentiation, glucagon-like peptide (GLP)-1 (1-37) or GLP-1 (7-36) (Sigma) was supplemented into single-cell cultures of adult pancreatic cells. During the culture period, cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. The number of epithelial-like colonies (ECs) was determined after 8 days of culture.

RT-PCR analysis. Detection of lineage-specific gene expression in each clonal colony by RT-PCR was conducted as described (23). Primer sequences are as follows (many have been presented already in our previous work [23,24]; those that have not are given in parentheses): α-cell marker preproglucagon and β-cell markers preproinsulin I, preproinsulin II, islet amyloid polypeptide (*IAPP*), and glucose transporter-2 (*glut-2*) (5′-ACA GAG CTA CAA TGC AAC GTG G-3′ and 5′-CAA CCA GAA TGC CAA TGA CGA T-3′); γ-cell marker PP (5′-CTC CCT GTT TCT CGT ATC CA-3′ and 5′-AGA GCA GGG AAT CAA GCC AA-3′); δ-cell marker preprosomatostatin, acinar cell markers amylase-2 and hormone-sensitive (HS) lipase, and ductal cell markers cytokeratin 19 and carbonic anhydrase II (5′-AAC GTT GAG TTT GAT GAC TCT-3′ and 5′-AGT TGT CCA CCA TCG CTT CTT-3′); and a miscellaneous gene nestin (5′-AGA AGAGCA GAA CTT AGA AT-3′ and 5′-TAG AGG TTT CAC AAT TCT CT-3′). Hepatocyte-related markers included albumin, α-fetoprotein, and glucose-6-phosphatase. Intestine/stomach-related markers included intestinal fatty-acid binding protein (*fabp-2*), gastric inhibitory peptide (*GIP*), cholecystokinin (*CCK*), tryptophan hydroxylase (*TPH*), gastrin (35), and pepsinogen F. Primers for transcriptional factors were also used as follows: pancreas duodenal homeobox (*Pdx*)-1, neurogenin 3 (*ngn3*) (5′-AGT GCT CAG TTC CAA TTC CAC-3′ and 5′-AAG AAG TCT GAG AAC ACC AGT-3′), hepatocyte nuclear factor (HNF)-1, HNF-3α, HNF-3β, HNF-3γ, HNF-4, and HNF-6 (5′-ATG ACC ATG GCC TGT GAA ACT-3′ and 5′-ATT CAG GTG GGC ATG AGG AT-3′). As a positive control, primers for hypoxanthine phosphoribosyltransferase (*HPRT*) were also prepared. PCR cycles were as follows: initial denaturation at 95°C for 4 min, followed by 35–45 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and final extension at 72°C for 10 min. PCR products were separated in 2% agarose gel.

Immunocytochemistry. Cells in each colony were cultured for 12 days. They were then washed three times with PBS and fixed first with 4% phosphate-buffered paraformaldehyde for 5 min at room temperature and then with 25% acetone in methanol for 1 min at room temperature. After the fixation, cells were washed in PBS containing 0.05% polyoxyethylene (20) sorbitan monolaurate (Tween 20) (Wako) and treated with 0.2% Triton X-100 (Sigma) for 1 h at room temperature. Nonspecific binding was blocked with 10% nonimmune serum of the species from which the secondary antibody had been obtained. Fixed cells were incubated with primary antibody goat anti-insulin (Santa

Cruz Biotechnology, Santa Cruz, CA), mouse anti-glucagon (Sigma), rabbit anti-somatostatin (Affiniti Research Products, Exeter, U.K.), or rabbit anti-amylase (Sigma) in a moist chamber for 16 h at 4°C. After washing in PBS-Tween 20 and blocking, cells were incubated with Alexa 488–conjugated donkey anti-goat IgG (Molecular Probes, Eugene, OR), Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), or Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for 3 h at 4°C, respectively. After final washing, cells were viewed using a Zeiss LSM510 laser scanning microscope.

Immunohistochemistry. Neonatal and adult pancreatic tissues were fixed in 4% phosphate-buffered paraformaldehyde overnight at 4°C, dehydrated overnight in 20% sucrose at 4°C, and then embedded in OCT compound. Cryostat sections of these tissues were dried and fixed secondarily by acetone for 10 min at room temperature. Sections were dried at −20°C overnight, washed with PBS-Tween 20, and treated with 0.2% Triton X-100 for 1 h at room temperature. For CA II and Flk-1 staining, tissues were treated with 10 mmol/l citric buffer (pH 6.0) for 1 h at 37°C between washing and Triton treatment. After washing in PBS-Tween 20 and blocking, tissues were incubated with the following primary antibodies in a moist chamber for 16 h at 4°C: goat anti-insulin, mouse anti-glucagon, goat anti-amylase (Santa Cruz Biotechnology), rabbit anti-Met (Santa Cruz Biotechnology), sheep anti-CAII (The Binding Site, Birmingham, U.K.), or goat anti-Flk-1 (Santa Cruz Biotechnology). As secondary antibodies, we used Alexa 488–conjugated goat anti-rabbit IgG (Molecular Probes), Alexa 488–conjugated donkey anti-goat IgG, Alexa 488–conjugated donkey anti-sheep IgG, Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories), and Cy3-conjugated donkey anti-rabbit IgG. Stained tissues were viewed using a Zeiss LSM510 laser scanning microscope.

Retroviral infection. Production of a retroviral vector is described elsewhere (24). For marking of a CAPC clone with enhanced green fluorescent protein (EGFP), concentrated virus supernatant was added to 6-well plate cultures in which cells had grown up to 40–50% confluence in 2.5 ml of standard medium with 5 μg/ml protamine sulfate (Sigma) followed by "spinoculation" (36). Residual virus was eliminated by washing the cells with PBS and changing the medium after 24 h. EGFP-positive cells were identified by FACS Vantage.

Cell transplantation. After the initiation of clonal culture, we maintained EGFP-tagged cells in culture by replating them every 7 days. Donor cells were usually prepared from cells obtained at these passage points. EGFP-tagged cells were trypsinized, washed, and resuspended at a concentration of 2×10^6 cells in 100 μl standard medium. They were then injected into pancreata of recipient mice through the common bile duct (C57BL/6, 4 weeks old, $n = 5$; Clea) under anesthesia. We also injected standard medium without cells as a negative control ($n = 5$).

RESULTS

Flow-cytometric isolation and enrichment of cells with high growth capacity in the neonatal pancreas. To examine the growth and differentiation potential of c-Met–positive cells in the pancreas, neonatal pancreata were dissociated and fractionated into four subpopulations by FACS, using antibodies to c-Met and c-Kit (stem cell factor receptor) after excluding hematopoietic cells identified by the expression of CD45 (leukocyte common antigen) and TER119 (a molecule resembling glycophorin and exclusively expressed on immature erythroid cells). Because Rachdi et al. (37) showed that c-Kit is expressed in some mature β-cells residing in islets, we attempted to eliminate such cells by using an antibody against c-Kit. Nonhematopoietic CD45$^-$ TER119$^-$ cells comprised 37.8 ± 4.11% of the initial population (mean ± SD). These cells were further subfractionated into the following groups: *1*) c-Met$^+$ c-Kit$^+$ cells (0.01 ± 0.02%), *2*) c-Met$^+$ c-Kit$^-$ cells (0.99 ± 0.11%), *3*) c-Met$^-$ c-Kit$^+$ cells (0.81 ± 0.07%), and *4*) c-Met$^-$ c-Kit$^-$ cells (35.9 ± 2.97%) (Fig. 1*A*). The proliferation of cells in each subpopulation was then examined in vitro.

We previously set up culture conditions (25) in which single cells proliferated to form clusters of up to several hundred cells. Sorted cells adhered to the culture dishes and formed clonal colonies as culture proceeded (Fig. 1*B–E*). Cells were initially cultured at 200 cells/cm^2, but only 20–30% of cells adhered to the culture dish; nonadherent cells were removed by changing the medium. This clonal density of the culture allowed colonies to form separately from each other. At day 8 of culture, colonies formed by morphologically identified epithelial-like cells (ECs) were seen (Fig. 1*F* and *G*). The cell that had initiated the formation of these colonies was provisionally designated as an EC-forming cell (ECFC). In addition to epithelial-like cells, fibroblast-like cells can grow, but not extensively. Fibroblast-like cells, however, do not express any marker genes for endocrine and exocrine pancreatic cells.

In in vitro colony assays of FACS-sorted cells, few ECs or none were found in CD45$^+$, TER119$^+$, c-Met$^+$ c-Kit$^+$ CD45$^-$ TER119$^-$, c-Met$^-$ c-Kit$^+$ CD45$^-$ TER119$^-$, and c-Met$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell populations. Most were found in c-Met$^+$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells. Sorting for c-Met$^+$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells achieved 13.1- and 9.4-fold enrichment of ECFCs compared with total neonatal pancreatic cells and CD45$^-$ TER119$^-$ cells, respectively (Fig. 1*H*).

Since c-Met is also expressed in vascular endothelial cells and HGF works as an angiogenic factor (38,39), we conducted further fractionation of the c-Met$^+$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell subpopulation into the following two distinct subpopulations by using an antibody against the vascular endothelial cell marker Flk-1: *1*) c-Met$^+$ Flk-1$^+$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells (0.26 ± 0.03%) and *2*) c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells (0.59 ± 0.01%) (Fig. 1*I*). In cultures with a clonal density of 200 cells/cm^2, ECFCs were identified mostly from the c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell subpopulation (Fig. 1*J*).

Differentiation potential of ECFCs. We next examined the differentiation potential of ECFCs. Cells in each EC were analyzed by RT-PCR to identify the expression of genes encoding several markers for pancreatic endocrine (α-cell, preproglucagon; β-cell, preproinsulin I and II; γ-cell, PP; and δ-cell, preprosomatostatin), acinar (amylase 2 and HS lipase), and ductal (cytokeratin 19 and carbonic anhydrase II) cells. Although it was difficult to find cells expressing these markers, aside from cytokeratin 19, before day 5 of culture, ECFCs exhibited various differentiation patterns at day 12 (Table 1). Most colonies contained two (21.4%; $n = 28$ colonies assessed), three (32.1%), four (28.6%), or five (17.9%) lineage cell types. Eleven colonies (39.3%) contained cells that were differentiating into all three major cell types: endocrine, acinar, and ductal. Immunocytochemical analysis also showed that ECs contained cells positive for insulin, glucagon, somatostatin, or amylase at day 12 (Fig. 2). These results indicate the multipotency of ECFCs.

Localization of c-Met–positive cells in the pancreas. In order to determine where c-Met–positive cells localize in the developing mouse pancreas, we performed immunohistochemical staining of neonatal pancreas with an anti-Met antibody. Positive cells were detected in acinar tissues (Fig. 3*A–C*) and also in some ducts (Fig. 3*D–F*) and vascular endothelia (Fig. 3*G–K*). The percentage of c-Met–positive cells that we estimate is <0.5% in acinar cells, <1% of ducts, and <10% of blood vessels. c-Met–positive ductal or vascular endothelial cells coexpressed either carbonic anhydrase II or Flk-1 (Fig. 3*D–F* and *I–K*). In

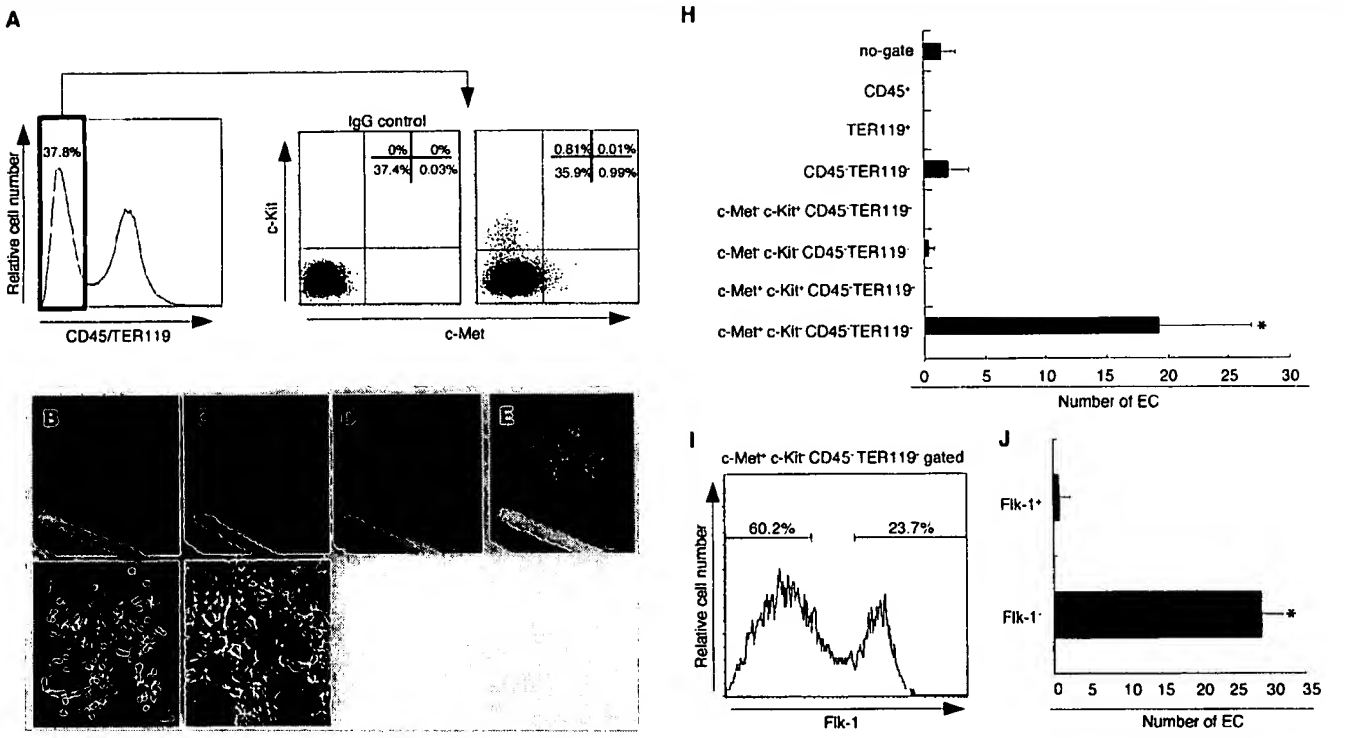


FIG. 1. Flow cytometric isolation and enrichment of colony-forming cells in the neonatal pancreas. *A*: CD45$^+$ or TER119$^+$ hematopoietic cells were gated and removed from the initial pancreatic tissue specimen. Nonhematopoietic (CD45$^-$ TER119$^-$) cells were fractionated based on c-Met and c-Kit expression. Percentages of fractionated cells are shown at the top of each panel. For in vitro colony assay, sorting gates were set for the c-Met$^-$ c-Kit$^+$, c-Met$^-$ c-Kit$^-$, c-Met$^+$ c-Kit$^+$, and c-Met$^+$ c-Kit$^-$ subpopulations. Establishment of the gate was based on the staining profiles of the negative control (IgG control). Representative data are shown for six independent experiments. *B–G*: Formation of a colony derived from a sorted cell in culture. Under the clonal density culture (200 cells/cm^2 in each 35-mm tissue culture dish), a single sorted cell attached itself to the culture dish after 12 h (*B*). The cell then began to divide and formed a colony after 48 (*C*), 72 (*D*), and 120 h (*E*). Scratches in the substrate serve to identify the field. *F* and *G*: A colony continued to grow, generating an EC at day 8. *H*: The number of ECs was counted after 8 days in the clonal density culture (200 cells/cm^2). *I*: We further fractionated the c-Met$^+$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell subpopulation into two subpopulations by using antibodies against a vascular endothelial cell marker Flk-1. For in vitro colony assay, sorting gates were set for the c-Met$^+$ Flk-1$^+$ c-Kit$^-$ CD45$^-$ TER119$^-$ and c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell subpopulations. *J*: The number of ECs was counted after 8 days in the clonal density culture (200 cells/cm^2). *H* and *J*: The graphs show the average of 18 dishes for each cell subpopulation in six independent experiments. *Mann-Whitney *U* test: $P < 0.001$. Scale bar: 100 μm.

contrast, c-Met–expressing cells in acinar tissues were not positive for any markers of pancreatic lineage cells (Fig. 3*C*, data not shown). In the human pancreas, it has been reported (27) that c-Met is expressed in β-cells, for which HGF acts as a mitogen. We detected some weakly c-Met–positive cells in mouse islets, but its expression was not consistent; it appeared to vary from location to location. Alternatively, c-Met expression within β-cells may have been specific to a particular strain or cellular developmental stage, occurring only during growth, differentiation, or functional maturity.

In the adult pancreas, stem/progenitor cells are thought to reside in the pancreatic ducts. Our present study shows that ductal cells, as well as some acinar cells, in the neonatal pancreas express c-Met and that they can be sorted using FACS. Therefore, we speculated that some

TABLE 1
Expression of various marker genes in independent neonatal epithelial colonies

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Preproglucagon (α-cell)	+	+	−	+	+	+	−	+	−	−	−	+	−	+	+	+	+	−	+	+	+	−	+	−	−	−	+	−
Preproinsulin I (β-cell)	+	+	−	−	+	+	−	+	−	−	+	−	+	−	+	−	+	−	+	+	+	−	−	+	−	+	+	+
Preproinsulin II (β-cell)	+	+	−	−	+	−	−	+	−	−	+	+	−	−	+	−	+	−	−	+	+	−	−	−	−	+	+	+
PP (γ-cell)	−	+	+	−	+	−	−	+	+	+	−	+	+	+	−	+	+	+	+	−	−	−	−	+	−	−	+	−
Preprosomatostatin (δ-cell)	−	−	−	−	+	−	−	−	−	−	−	−	+	+	+	−	+	−	−	−	−	−	+	+	−	−	−	−
Amylase 2 (exocrine cell)	−	−	+	+	−	−	+	−	−	−	−	+	+	+	−	−	−	−	−	+	+	+	+	+	−	+	+	−
HS lipase (exocrine cell)	−	−	+	−	−	−	+	−	−	−	−	+	−	−	−	−	−	+	−	+	−	+	+	−	+	−	−	+
Cytokeratin 19 (ductal cell)	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	+
Carbonic anhydrase II (ductal cell)	−	−	+	−	−	−	+	−	−	−	−	+	+	−	−	−	−	−	+	+	−	+	+	−	+	−	−	−
c-Met	−	+	+	−	+	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+
HPRT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lineages included in a colony	3	3	3	3	5	3	2	4	2	2	2	5	5	5	4	3	5	3	4	4	4	2	4	4	2	3	4	3

HPRT, hypoxanthine phosphoribosyltransferase.

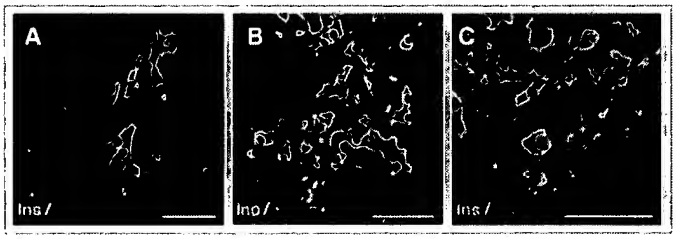


FIG. 2. In vitro multilineage colony formation from sorted c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells. c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells were sorted, cultured for 12 days, and allowed to form ECs. Then, immunocytochemical double staining was conducted on EC constituent cells. *A*: Insulin (green) and glucagon (red). *B*: Insulin (green) and somatostatin (red). *C*: Insulin (green) and amylase (red). Scale bar: 50 μm (*A–C*).

ductal cells in the adult pancreas would also be positive for c-Met. Expectedly, immunohistochemical analysis revealed that c-Met is expressed in adult pancreas in similar regions as seen for neonatal tissues, including ductal, acinar, and vascular endothelial cells (Fig. 3*L–Q*). These data show that the expression of c-Met is maintained in some restricted regions from developing through the adult stage, suggesting that c-Met–positive cells in the adult pancreas would possess stem/progenitor cell characteristics, similar to neonatal c-Met–positive cells.

Isolation and characterization of c-Met–positive cells in adult mouse pancreas. We next tried to isolate c-Met–positive cells from the adult pancreas and examine the growth and differentiation potential of these cells. Cells obtained from the adult pancreas were fractionated by FACS using antibodies against c-Met, Flk-1, c-Kit, CD45, and TER119 (Fig. 4*A*). Similar to neonates, sorting of c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells in the adult pancreas achieved high enrichment of adult ECFCs (Fig. 4*B*). Although a few cells exhibited autofluorescence, they do not form colonies in culture. High enrichment of ECFCs permitted efficient culture of clone-sorted c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells for analyses of their differentiation potential. Cells identified upon clone sorting by flow cytometry were cultured in individual wells of 96-well plates. Each well was examined under a microscope after clone sorting to confirm the deposition of a single cell. Once a cell sorter is adjusted for optimal setting before the experiment, we seldom find wells with more than two cells after clone sorting. When we found these wells, we excluded them from samples for analysis.

To examine the differentiation potential of adult ECFCs, immunocytochemistry and RT-PCR were performed on EC constituent cells. They strongly express the ductal cell marker cytokeratin 19; however, no insulin, glucagon, somatostatin, or amylase expression was detected (Fig. 4*C* and *D*, data not shown). RT-PCR analysis also revealed that all examined ECs contained cells positive for CK19 and c-Met, but none expressed any endocrine cell markers (Table 2). However, *Pdx-1*, expressed in differentiating ductal cells, and an acinar cell marker, HS lipase, were expressed in cells from several ECs. These results suggest that supplemental factors would be required for such cells to differentiate into functional cells. Therefore, we sought to induce lineage marker expression in isolated cells using GLP-1, especially GLP-1 (7-36) and GLP-1 (1-37), which can induce differentiation of β-cells in the pancreas (40) or activate insulin gene expression in intestinal epithelial cells (41), respectively. In cultures with these peptides,

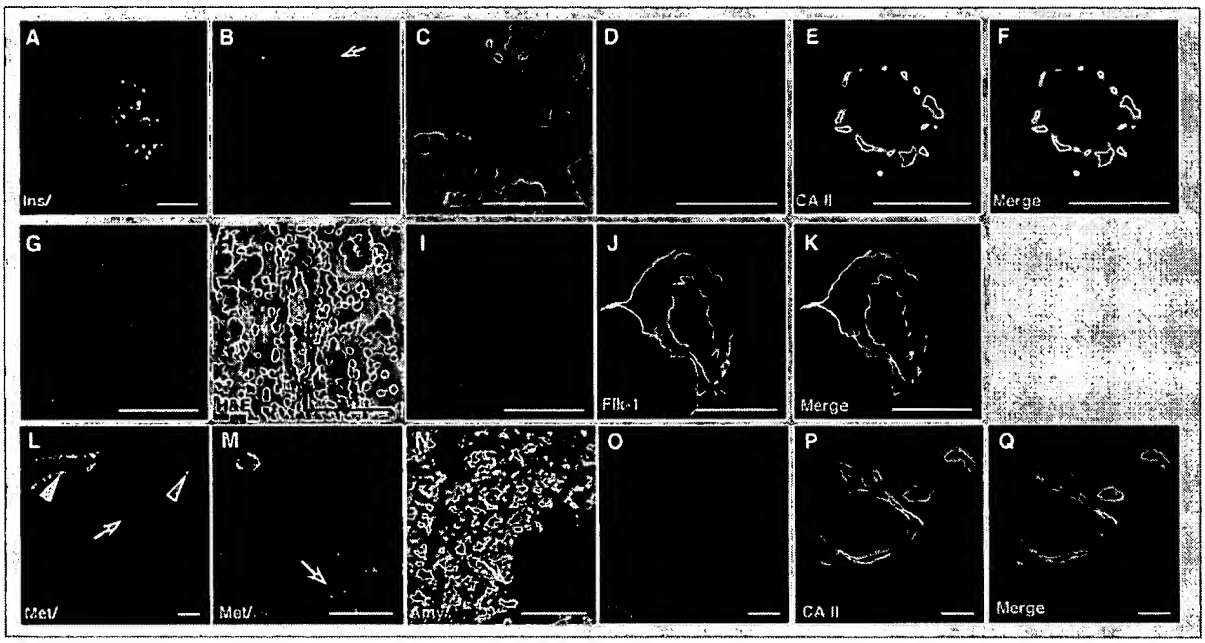


FIG. 3. Localization of c-Met–positive cells in the mouse pancreas. *A–C*: In the neonatal pancreas, a few c-Met–positive cells resided in acinar tissues (*B*, arrow), but not in islets. Note that c-Met was detected in cells expressing no endocrine or acinar cell markers. *A*: Insulin (green) and amylase (red). *B*: A serial section of *A* was stained by anti–c-Met antibody (red, shown as arrow). *C*: A serial section of *A* was stained by antibodies against amylase (green) and c-Met (red). *D–F*: c-Met–positive cells were found in some ducts. *D*: c-Met (red). *E*: CAII (green). *F*: Merge. *G–K*: Cells residing in several vascular endothelia also expressed c-Met. *G* and *I*: c-Met (red). *H*: Hematoxylin and eosin stain. *J*: Flk-1 (green). *K*: Merge. *L–N*: In the adult pancreas, c-Met is expressed in ductal cells (*L*, arrow), vascular endothelial cells (*L*, arrowheads), and cells residing around acinar tissues (*M* and *N*, arrow). Note that these immunoreactive regions for c-Met are maintained from the neonatal through the adult stage. *O–Q*: c-Met–positive ductal cells in the adult pancreas also expressed the ductal cell marker CAII. *O*: c-Met (red). *P*: CAII (green). *Q*: Merge. Scale bar: 100 μm (*A*, *B*, *G*, *H*, and *L–N*), 50 μm (*D–F* and *I–K*), 25 μm (*C*), and 10 μm (*O–Q*).

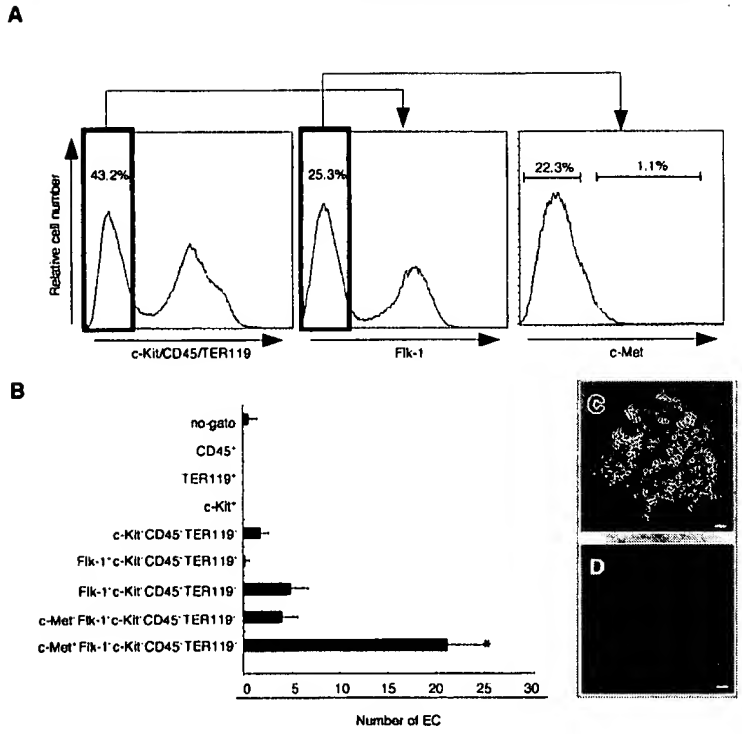


FIG. 4. Flow cytometric isolation and enrichment of colony-forming cells in the adult pancreas. *A*: After CD45$^+$, TER119$^+$, or c-Kit$^+$ cells from the adult pancreas were gated out, c-Kit$^-$ CD45$^-$ TER119$^-$ cells were fractionated by Flk-1 expression, and finally Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells were further fractionated by c-Met expression. Percentages of the fractionated cells are shown at the top of each panel. Establishment of the gate was based on the staining profiles of the negative control. Representative data are shown for six independent experiments. *B*: The number of adult ECs was counted after 8 days in the clonal density culture (10 cells/cm^2). This graph shows the average of 18 dishes for each cell subpopulation from six independent experiments. *C* and *D*: Formation of a clonal colony derived from a sorted c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell 2 weeks after the initiation of single cell culture. Almost all colony-constituent cells were positive for the ductal cell marker cytokeratin 19. *P < 0.001. Scale bar: 100 μm.

expression of several marker genes, including insulin genes, emerged in cells derived from some ECFCs (Table 2). These data suggest that c-Met is a convenient cell surface marker of cells with high proliferative capacity and multiple differentiation potential in adult pancreas, and it is useful to isolate such cell populations by using FACS to examine their characteristics clonally. Since our culture environment, however, is still not sufficient to induce complete differentiation of adult ECFCs into endocrine and acinar cells, further investigation of their potential for differentiation is required.

Cell expansion from sorted neonatal c-Met–positive cells. When we cultured no-gated cells derived from neonatal pancreas at high density (1,000 cells/cm^2), several epithelial-like cells proliferated, reaching semiconfluency after 1 month in culture. They also produced cell aggregates that mimicked pancreatic islet structures. We provisionally designated cells that produce such aggregates as CAPCs. Ramiya et al. (6) reported that these islet-like structures were generated from stem cells derived from ductal epithelia as culture proceeded. Although the culture conditions were different, similar cells also appeared in our culture condition. To determine which cell subpopulation contained these CAPCs in vivo, we cultured directly sorted cells from neonatal pancreas using antibodies for c-Met, Flk-1, c-Kit, CD45, and TER119. When we cultured cells at low density (<200 cells/cm^2), it was difficult to find cells 1 month later. However, in middle- to high-density culture conditions (500–1,000 cells/cm^2), cells sorted into no-gated, CD45$^-$ TER119$^-$, c-Met$^+$ c-Kit$^-$ CD45$^-$ TER119$^-$, and c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell subpopulations proliferated and gen-

TABLE 2
Expression of various marker genes in independent adult epithelial colonies in single cell culture

	HGF + EGF										HGF + EGF + GLP-1 (1–37)					HGF + EGF + GLP-1 (7–36)				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Preproglucagon (α-cell)	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	+	+
Preproinsulin I (β-cell)	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	+	+	−
Preproinsulin II (β-cell)	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	+	−
PP (γ-cell)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Preprosomatostatin (δ-cell)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Amylase 2 (exocrine cell)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
HS lipase (exocrine cell)	−	−	−	+	−	+	−	−	−	−	−	+	+	−	+	+	−	+	+	−
Cytokeratin 19 (ductal cell)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carbonic anhydrase II (ductal cell)	+	−	−	+	−	+	−	+	−	−	−	−	+	+	+	+	−	+	+	+
Pdx1	+	−	−	−	+	+	−	−	−	−	−	+	+	+	−	+	−	+	+	−
c-Met	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HPRT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lineages included in a colony	1	1	1	2	1	2	1	1	1	1	1	2	4	2	3	4	1	3	4	2

HPRT, hypoxanthine phosphoribosyltransferase.

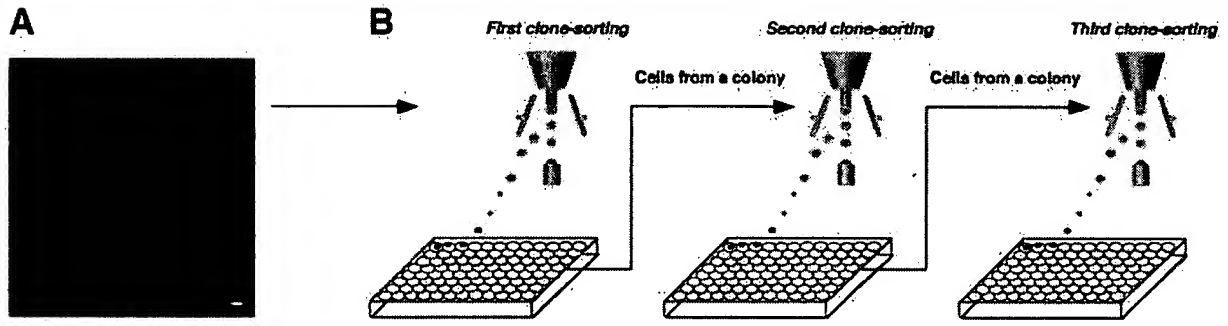


FIG. 5. Clonal analysis of CAPCs derived from c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ sorted cells. *A*: Islet-like cell aggregates that budded from the monolayer cell sheet were observed in long-term cultures of neonate-derived c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells (arrowheads). *B*: The CAPCs were then clone sorted by FACS and individually cultured in 96-well tissue culture plates. Second and third clone sortings were also conducted by using cells included in a clonal colony. Scale bar: 50 μm.

erated cell aggregates (Fig. 5*A*). No cells were found when we cultured cells from other cell subpopulations, even at much higher-density conditions (>10,000 cells/cm^2). These results show that CAPCs express c-Met but not Flk-1, c-Kit, CD45, or TER119 in the neonatal pancreas.

To characterize these cells clonally, we conducted single-pass sorting by FACS and cultured them in individual wells of 96-well plates (Fig. 5*B*, first clone sorting). Colonies were formed by 3.13 ± 1.04% of sorted CAPCs (average of six plates [576 wells], three independent experiments). Among the progeny of a single cell, the expression of genes characteristic of not only pancreatic endocrine and acinar lineages, but also for progenitor cells (*Pdx-1*, *ngn3*, and *nestin*), was detectable (Table 3).

TABLE 3
Expression of various marker genes in independent colonies after serial clone sorting of CAPCs

	First clone sorting					Second clone sorting					Third clone sorting				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Pancreas-related genes															
Preproglucagon	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Preproinsulin I	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
Preproinsulin II	+	−	−	+	+	−	+	+	+	+	+	+	−	+	+
IAPP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
glut-2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Preprosomatostatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amylase 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HS lipase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cytokeratin 19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carbonic anhydrase II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nestin	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+
c-Met	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hepatocyte-related genes															
Albumin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-Tetoprotein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose-6-phosphatase	+	+	+	−	+	+	−	−	+	+	+	+	−	+	+
Intestine/stomach-related genes															
Fabp-2	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
GIP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CCK	+	+	−	+	+	+	+	+	−	+	+	−	+	+	+
TPH	−	+	−	−	+	+	−	+	−	+	+	+	+	+	−
Pepsinogen F	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
Gastrin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Transcription factor genes															
Pdx1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ngn3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HNF-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HNF-3α	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HNF-3β	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HNF-3γ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HNF-6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HPRT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

CCK, cholecystokinin; GIP, gastric inhibitory peptide; HPRT, hypoxanthine phosphoribosyltransferase; IAPP, islet amyloid polypeptide; TPH, tryptophan hydroxylase.

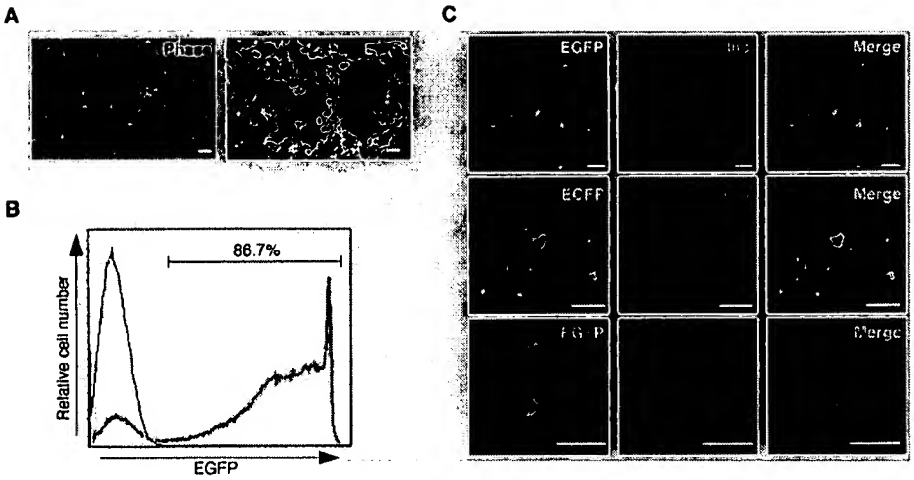


FIG. 6. Clonally expanding CAPCs in culture can differentiate into pancreatic endocrine and acinar cells in vivo. *A*: For use as donor cells, an expanding CAPC clone was marked genetically with EGFP by retrovirus infection. *B*: After a single round of infection, FACS analysis revealed that 85.4 ± 4.2% (mean ± SD) of cells highly expressed EGFP (n = 3). *C*: One month after transplantation of cells, the pancreata of recipient mice were fixed and sectioned. In islets, several EGFP-positive cells expressing the β-cell marker insulin or the α-cell marker glucagon were observed. An acinar cell marker amylase was also detected in EGFP-positive cells residing in acinar tissues, showing that donor cells had given rise to both endocrine and acinar cells in vivo and reconstituted these tissues with host cells. Scale bar: 100 μm (*A*) and 40 μm (*C*).

Furthermore, the expression of hepatocyte- and intestinal/stomach-related genes was also detected (Table 3). To examine whether CAPCs expanded with self-renewing cell divisions, we conducted subcloning experiments for cells in a CAPC-derived colony (Fig. 5*B*, *second and third clone sorting*). In Table 3, secondary formed colonies (colony number 6–10) and tertiary formed colonies (colony number 11–15) were derived from cells in the colonies numbered 1 and 6, respectively. RT-PCR analysis showed that various endoderm marker genes were expressed in colonies formed by second and third single-cell sorting, similar to primary formed colonies (Table 3). These data indicate that long-term cultured c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells can differentiate into multiple cell types in endodermal organs, while they maintain multipotent cells by self-renewing cell divisions.

In vivo differentiation of clonally expanding CAPCs. In order to determine whether a clonally expanding CAPC in culture could differentiate into both pancreatic endocrine and acinar cells in vivo after transplantation, we injected them through the common bile duct into a recipient pancreas. Cells derived from colony 1 (Table 3) were used for this in vivo analysis. Before cell transplantation, donor cells were marked genetically with EGFP by retrovirus infection to distinguish donor cells from recipient cells (Fig. 6*A*). The concentrated vesicular stomatitis virus pseudotyped retrovirus allowed high transduction frequencies, and >85% of cells highly expressed EGFP after a single round of infection (Fig. 6*B*).

At 1 month posttransplantation, all five recipient mice exhibited donor cell integration into pancreatic tissues. Staining for the β-cell marker insulin, α-cell marker glucagon, and acinar cell marker amylase showed that donor cells had given rise to both endocrine and acinar cells in vivo (Fig. 6*C*). However, engrafted cells were widely scattered in the pancreas, and few of them existed with recipient α- and β-cells in islets. Integration and engraftment of donor cells in islets might require an adequate tissue injury for islet neogenesis. Because the engraftment of donor cells was slightly found, it is also required to improve the protocol of cell transplantation and to prepare a number of transplantable functional cells to realize the repopulation of islet and acinar tissues. This result, however, is the first report to show that derivatives of a single cell can survive in the pancreas for at least 1 month and differentiate into endocrine and acinar lineage cells in vivo as well as in vitro.

DISCUSSION

A previous study (6) has shown that cells derived from ductal epithelia generated islet-like cell aggregates and expressed c-Met. Because such ductal cell fractions, however, still contained many other kinds of cells, c-Met has not been defined as a possible marker to characterize stem/progenitor cells in the pancreas. Our present results indicate that c-Met is a useful marker to prospectively identify and isolate cells that fulfill the criteria of pancreatic stem/progenitor cells. By sorting c-Met–positive cells using FACS in this study, we achieved prospective isolation of a rare c-Met–positive cell population by completely excluding other lineage cells and revealed their high proliferative capacity and multiple differentiation potential. This stem/progenitor cell activity is restricted to the c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells, which constitute scarcely ~1% of both the neonatal and adult pancreas. The remaining 99% of the cells, in contrast, have much less activity of stem/progenitor cells. Furthermore, when neonatal c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells were cultured for longer periods (>1 month), a semiconfluent monolayer of cells appeared, from which islet-like cell aggregates budded out, and they gave rise to cells expressing multiple lineage markers for endodermal digestive organs in vitro. These findings suggest that c-Met–positive cells residing in the pancreas possess potential for wider cell lineage plasticity in organs of endoderm origin or that they are more primitive endodermal stem cells.

The c-Met/HGF interaction mediated by signaling between mesenchymal and epithelial cells plays important roles in pancreatic development (27,28). Whether this essential signaling is directly responsible for stem cell activity, however, remains to be determined. In this study, we showed that some ductal and acinar cells in the neonatal pancreas are immunoreactive for c-Met and that they could be specifically separated from other differentiated cells by using FACS. These sorted c-Met–positive cells proliferated exclusively in response to HGF and exhibited multipotent differentiation. These findings permit us to speculate that the c-Met/HGF interaction is critically responsible for stem cell growth and differentiation in the developing pancreas. Interestingly, our studies

showed that nestin, a recently reported (42) marker for stem-like cells that reside in ductal epithelia and islets, was expressed in both CAPCs (Table 3) and EC constituent cells (data not shown). Nestin, however, was also detected in the c-Met–negative cell fraction, suggesting that this antigen is expressed in a much broader cell population, including differentiated cells.

In the adult pancreas, immunohistochemical analysis showed expression of c-Met in several ductal cells. Also, most adult ECs derived from sorted c-Met–positive cells possessed ductal cell characteristics, such as expression of CK19, and they received a growth signal from HGF to grow in culture. These results indicate that adult ECFCs reside in some pancreatic ducts, express c-Met, and are capable of forming colonies in response to HGF after sorting. Therefore, the c-Met/HGF interaction, in addition to its role in pancreatic development, would be crucial for homeostatic cell turnover and regeneration from stem/progenitor cells residing in the adult pancreas. Our data also demonstrate that the multilineage differentiation capacity of ECFCs is much more strictly regulated in adults than in neonates. For this reason, neonatal "activating" stem cells, rather than those isolated from the adult pancreas, would be more suitable for clinical applications, such as cell transplantation, that require the production of multiple cell type lineages and self-renewing cell proliferation. Although most adult ECs possess a ductal phenotype, several colonies included cells expressing *Pdx-1* or the acinar cell marker HS lipase, suggesting that the original ECFC could potentially differentiate into both endocrine and acinar lineage cells. In fact, GLP-1 can induce expression of several marker genes in cells derived from adult ECFCs. However, this effect is not sufficient to produce many functional cells for therapeutic application. Differences of differentiation potential between c-Met–positive cells in the neonatal and adult pancreas may indicate that adult "silencing" stem cells residing in ducts cannot respond immediately to severe β-cell loss. Instead of these cells, intraislet β-cell progenitors, which emerge in islets following depletion of the resident cell population by a β-cell toxin (e.g., streptozotocin), may differentiate to recover insulin content in β-cell neogenesis (43). Screening for differentiation-inducing factors using our clonal cell culture system for stem/progenitor cells should identify a critical substrate that activates differentiation of endogenous PSCs and efficient reproduction of functional β-cells.

A candidate mouse hepatic stem cell, hepatic colony-forming unit in culture (H-CFU-C), which has been reported previously (24) by our group, has a similar phenotype to a candidate PSC described here, including the expression of c-Met but not c-Kit, CD45, and TER119. The expression of CD49f (α6 integrin subunit), which is weakly expressed in H-CFU-Cs, is also detected in most c-Met–positive pancreatic cells. Furthermore, both cell types have the potential to differentiate into hepatic, pancreatic, gastric, and intestinal lineage cells in vitro. These findings suggest that pluripotent cells, like H-CFU-C in the fetal liver, are also present in the pancreas. However, these putative hepatic and pancreatic stem cells are not completely equivalent, as they possess several striking differences. For example, several extracellular matrices that exert strong growth induction of H-CFU-C do not have an effect on the ability of a candidate PSC to proliferate in culture (23). For this reason, both cell types should not be characterized as the same cell population. Our present data and several previous reports (42,44–48), however, suggest that hepatic or endodermal stem cells may persist in postnatal pancreatic tissue. In particular, it was reported (44,45,47) that pancreatic hepatocytes could develop from pancreatic ductal and/or acinar cells. These regions were defined in this study as being immunoreactive for c-Met and contained candidate PSCs in both neonates and adults. Alternatively, Wang et al. (49) showed that pancreatic cells capable of significant liver reconstitution are not derived from the ductal pancreas. Although further intensive study is required to identify the basis for pluripotency in these cells, it is possible to speculate that pluripotent stem cells for endodermal digestive organs have common phenotypic and physiological characteristics and are uniformly present in the liver, pancreas, stomach, intestine, and other endoderm-derived organs.

As a result of cell transplantation, clonally cultured CAPCs were capable of engrafting in the recipient pancreas and differentiating into endocrine and acinar cells. We infected donor cells with a retrovirus and marked them for EGFP expression in vitro to trace them after transplantation. Single-virus infection reproducibly achieved a high tagging efficiency, suggesting that gene-modified cells are theoretically useful for clinical cell-replacement therapy for the treatment of diabetes. Although insulin-positive donor-derived cells were also found when we transplanted CAPCs derived from EGFP transgenic mice (A.S., unpublished data), the possibility of cell fusion, uptake of DNA from dead cells, or reactivation of the retrovirus after cell transplantation cannot be completely ruled out. Further examination is required to address this issue and to achieve efficient differentiation and replacement of recipient pancreatic tissues by CAPCs.

Purification and characterization of PSCs will allow us to determine their full developmental potential by examining sorted stem cells directly. For this experimental goal, further enrichment of PSCs must be conducted by combining several antibodies with the anti–c-Met antibody, and culture conditions should be improved for clonogenic expansion of directly sorted cells. Manipulation of such cells may accelerate their proliferation and differentiation, make them able to generate functional β-cells more efficiently, and enhance hormone secretion from other endocrine lineage cells. Prospective identification, direct sorting, selective expansion, and precise differentiation control of pluripotent stem cells could provide a means of successful islet cell transplantation in patients with diabetes due to absolute or relative loss of β-cells.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan (14207046, 14081201, and 14657279) and by JSPS Research Fellowships for Young Scientists. This work was also supported by grants from The Mitsubishi Foundation and the Nissan Science Foundation and by grants for the leading project from the Ministry of Education, Science and Culture of Japan.

We thank N. Ukawa and Y. Jinzenji for technical support and Y. Morita for FACS operation.

REFERENCES

1. Pictet RL, Rutter WJ: Development of the embryonic endocrine pancreas. In *Handbook of Physiology*. Steiner DF, Frenkel N, Eds. Washington DC, Williams and Wilkins, 1972, p. 25–66
2. Pictet RL, Clark WR, Williams RH, Rutter WJ: An ultrastructural analysis of the developing embryonic pancreas. *Dev Biol* 29:436–467, 1972
3. Githens S: The pancreatic duct cell: proliferative capabilities, specific characteristics, metaplasia, isolation, and culture. *J Pediatr Gastroenterol Nutr* 7:486–506, 1988
4. Slack JM: Developmental biology of the pancreas. *Development* 121:1569–1580, 1995
5. Cornelius JG, Tchernev V, Kao KJ, Peck AB: In vitro-generation of islets in long-term cultures of pluripotent stem cells from adult mouse pancreas. *Horm Metab Res* 29:271–277, 1997
6. Ramiya VK, Maraist M, Arfors KE, Schatz DA, Peck AB, Cornelius JG: Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. *Nat Med* 6:278–282, 2000
7. Bonner-Weir S, Taneja M, Weir GC, Tatarkiewicz K, Song KH, Sharma A, O'Neil JJ: In vitro cultivation of human islets from expanded ductal tissue. *Proc Natl Acad Sci U S A* 97:7999–8004, 2000
8. Temple S: Division and differentiation of isolated CNS blast cells in microculture. *Nature* 340:471–473, 1989
9. Davis AA, Temple S: A self-renewing multipotential stem cell in embryonic rat cerebral cortex. *Nature* 372:263–266, 1994
10. Leary T, Jones PL, Appleby M, Blight A, Parkinson K, Stanley M: Epidermal keratinocyte self-renewal is dependent upon dermal integrity. *Invest Dermatol* 99:422–430, 1992
11. Jones PH, Harper S, Watt FM: Stem cell patterning and fate in human epidermis. *Cell* 80:83–93, 1995
12. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR: Multilineage potential of adult human mesenchymal stem cells. *Science* 284:143–147, 1999
13. Baroffio A, Hamann M, Bernheim L, Bochaton-Piallat ML, Gabbiani G, Bader CR: Identification of self-renewing myoblasts in the progeny of single human muscle satellite cells. *Differentiation* 60:47–57, 1996
14. Tropepe V, Coles BL, Chiasson BJ, Horsford DJ, Elia AJ, McInnes RR, van der Kooy D: Retinal stem cells in the adult mammalian eye. *Science* 287:2032–2036, 2000
15. Spangrude GJ, Heimfeld S, Weissman IL: Purification and characterization of mouse hematopoietic stem cells. *Science* 241:58–62, 1988
16. Osawa M, Hanada K, Hamada H, Nakauchi H: Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* 273:242–245, 1996
17. Taniguchi H, Toyoshima T, Fukao K, Nakauchi H: Presence of hematopoietic stem cells in adult liver. *Nat Med* 2:198–203, 1996
18. Matthews W, Jordan CT, Wiegand GW, Pardoll D, Lemischka IR: A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. *Cell* 65:1143–1152, 1991
19. Phillips RL, Ernst RE, Brunk B, Ivanova N, Mahan MA, Deanehan JK, Moore KA, Overton GC, Lemischka IR: The genetic program of hematopoietic stem cells. *Science* 288:1635–1640, 2000
20. Morrison SJ, White PM, Zock C, Anderson DJ: Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells. *Cell* 5:737–749, 1999
21. Rietze RL, Valcanis H, Brooker GF, Thomas T, Voss AK, Bartlett PF: Purification of a pluripotent neural stem cell from the adult mouse brain. *Nature* 412:736–739, 2001
22. Kubota H, Reid LM: Clonogenic hepatoblasts, common precursors for hepatocytic and biliary lineages, are lacking classical major histocompatibility complex class I antigen. *Proc Natl Acad Sci U S A* 97:12132–12137, 2000
23. Suzuki A, Zheng YW, Kondo R, Kusakabe M, Takada Y, Fukao K, Nakauchi H, Taniguchi H: Flow cytometric separation and enrichment of hepatic progenitor cells in the developing mouse liver. *Hepatology* 32:1230–1239, 2000
24. Suzuki A, Zheng YW, Kaneko S, Onodera M, Fukao K, Nakauchi H, Taniguchi H: Clonal identification and characterization of self-renewing pluripotent stem cells in the developing liver. *J Cell Biol* 156:173–184, 2002
25. Suzuki A, Oyama K, Fukao K, Nakauchi H, Taniguchi H: Establishment of clonal colony-forming assay system for pancreatic stem/progenitor cells. *Cell Transplant* 11:451–453, 2002
26. Sonnenberg E, Meyer D, Weidner KM, Birchmeier C: Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. *J Cell Biol* 123:223–235, 1993
27. Otonkoski T, Cirulli V, Beattie M, Mally MI, Soto G, Rubin JS, Hayek A: A role for hepatocyte growth factor/scatter factor in fetal mesenchyme-induced pancreatic beta-cell growth. *Endocrinology* 137:3131–3139, 1996
28. Beattie GM, Rubin JS, Mally MI, Otonkoski T, Hayek A: Regulation of proliferation and differentiation of human fetal pancreatic islet cells by extracellular matrix, hepatocyte growth factor, and cell-cell contact. *Diabetes* 45:1223–1228, 1996
29. Furukawa T, Duguid WP, Kobari M, Matsuno S, Tsao MS: Hepatocyte growth factor and Met receptor expression in human pancreatic carcinogenesis. *Am J Pathol* 147:889–895, 1995
30. Kiehne K, Herzig KH, Folsch UR: C-met expression in pancreatic cancer and effects of hepatocyte growth factor on pancreatic cancer cell growth. *Pancreas* 15:35–40, 1997
31. Otte JM, Kiehne K, Schmitz F, Folsch UR, Herzig KH: C-met protooncogene expression and its regulation by cytokines in the regenerating pancreas and in pancreatic cancer cells. *Scand J Gastroenterol* 35:90–95, 2000
32. Ohlsson B, Jansen C, Ihse I, Axelson J: Epidermal growth factor induces cell proliferation in mouse pancreas and salivary glands. *Pancreas* 14:94–98, 1997
33. Miettinen PJ, Huotari M, Koivisto T, Ustinov J, Palgi J, Rasilainen S, Lehtonen E, Keski-Oja J, Otonkoski T: Impaired migration and delayed differentiation of pancreatic islet cells in mice lacking EGF-receptors. *Development* 127:2617–2627, 2000
34. Cras-Meneur C, Elghazi L, Czernichow P, Scharfmann R: Epidermal growth factor increases undifferentiated pancreatic embryonic cells in vitro: a balance between proliferation and differentiation. *Diabetes* 50:1571–1579, 2001
35. Jensen J, Pedersen EE, Galante P, Hald J, Heller RS, Ishibashi M, Kageyama R, Guillemot F, Serup P, Madsen OD: Control of endodermal endocrine development by Hes-1. *Nat Genet* 24:36–44, 2000
36. Kotani H, Newton PB 3rd, Zhang S, Chiang YL, Otto E, Weaver L, Blaese RM, Anderson WF, McGarrity GJ: Improved methods of retroviral vector transduction and production for gene therapy. *Hum Gene Ther* 5:19–28, 1994
37. Rachdi L, El Ghazi L, Bernex F, Panthier JJ, Czernichow P, Scharfmann R: Expression of the receptor tyrosine kinase KIT in mature β-cells and in the pancreas in development. *Diabetes* 50:2021–2028, 2001
38. Nakamura Y, Morishita R, Higaki J, Kida I, Aoki M, Moriguchi A, Yamada K, Hayashi SI, Yo Y, Matsumoto K, Nakamura T Ogihara T: Expression of local hepatocyte growth factor system in vascular tissues. *Biochem Biophys Res Commun* 215:483–488, 1995
39. Kuba K, Matsumoto K, Ohnishi K, Shiratsuchi T, Tanaka M, Nakamura T: Kringle 1-4 of hepatocyte growth factor inhibits proliferation and migration of human microvascular endothelial cells. *Biochem Biophys Res Commun* 279:846–852, 2000
40. Perfetti R, Zhou J, Doyle ME, Egan JM: Glucagon-like peptide-1 induces cell proliferation and pancreatic-duodenum homeobox-1 expression and increases endocrine cell mass in the pancreas of old, glucose-intolerant rats. *Endocrinology* 141:4600–4605, 2000
41. Suzuki A, Nakauchi H, Taniguchi H: Glucagon-like peptide 1 (1-37) converts intestinal epithelial cells into insulin-producing cells. *Proc Natl Acad Sci U S A* 100:5034–5039, 2003
42. Zulewski H, Abraham EJ, Gerlach MJ, Daniel PB, Moritz W, Muller B, Vallejo M, Thomas MK, Habener JF: Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. *Diabetes* 50:521–533, 2001
43. Fernandes A, King LC, Guz Y, Stein R, Wright CV, Teitelman G: Differentiation of new insulin-producing cells is induced by injury in adult pancreatic islets. *Endocrinology* 138:1750–1762, 1997
44. Scarpelli DG, Rao MS: Differentiation of regenerating pancreatic cells into hepatocyte-like cells. *Proc Natl Acad Sci U S A* 78:2577–2581, 1981
45. Rao MS, Dwivedi RS, Yeldandi AV, Subbarao V, Tan XD, Usman MI, Thangada S, Nemali MR, Kumar S, Scarpelli DG: Role of periductal and ductular epithelial cells of the adult rat pancreas in pancreatic hepatocyte lineage: a change in the differentiation commitment. *Am J Pathol* 134: 1069–1086, 1989
46. Bisgaard HC, Thorgeirsson SS: Evidence for a common cell of origin for primitive epithelial cells isolated from rat liver and pancreas. *J Cell Physiol* 147:333–343, 1991
47. Sirica AE: Ductular hepatocytes. *Histol Histopathol* 10:433–456, 1995
48. Chen JR, Tsao MS, Duguid WP: Hepatocytic differentiation of cultured rat pancreatic ductal epithelial cells after in vivo implantation. *Am J Pathol* 147:707–717, 1995
49. Wang X, Al-Dhalimy M, Lagasse E, Finegold M, Grompe M: Liver repopulation and correction of metabolic liver disease by transplanted adult mouse pancreatic cells. *Am J Pathol* 158:571–579, 2001

Leukemia (2007) 21, 860–867

www.nature.com/leu

H

REVIEW

A hypothesis for an embryonic origin of pluripotent Oct-4$^+$ stem cells in adult bone marrow and other tissues

MZ Ratajczak[1], B Machalinski[2], W Wojakowski[3], J Ratajczak[1] and M Kucia[1]

[1]*Stem Cell Biology Program at James Graham Brown Cancer Center, University of Louisville, Louisville, KY, USA;* [2]*Department of Physiology, Pomeranian Medical University, Szczecin, Poland and* [3]*Third Department of Cardiology, Medical University of Silesia, Katowice, Poland*

Accumulating evidence demonstrates that adult tissues contain a population of stem cells that express early developmental markers such as stage-specific embryonic antigen and transcription factors Oct-4 and Nanog. These are the markers characteristic for embryonic stem cells, epiblast stem cells and primordial germ cells. The presence of these stem cells in adult tissues including bone marrow, epidermis, bronchial epithelium, myocardium, pancreas and testes supports the concept that adult tissues contain some population of pluripotent stem cells that is deposited in embryogenesis during early gastrulation. In this review we will discuss these data and present a hypothesis that these cells could be direct descendants of the germ lineage. The germ lineage in order to pass genes on to the next generations creates soma and thus becomes a 'mother lineage' for all somatic cell lineages present in the adult body.
Leukemia (2007) **21,** 860–867. doi:10.1038/sj.leu.2404630;
published online 8 March 2007
Keywords: Oct-4; Nanog; SSEA-4; VSELs; embryonic stem cells

'I feel quite optimistic about the possibility of someone discovering dormant embryonic totipotent stem cells in very small number, in all normal tissues....'

Dr Emilia Frindel, May 2000

Introduction

Stem cells are endowed with the property of self-renewal and the ability to differentiate into cells that are committed to restricted developmental pathways. The compartment of stem cells is organized in a hierarchical manner from the (i) most primitive (totipotent) stem cells that are able to form both embryo and placenta, (ii) pluripotent stem cells (PSC) that are able only to form the embryo but has lost the capacity to form the trophoblast (which gives rise to the placenta), (iii) multipotent stem cells in particular three germ layers (endo-, meso- and ectoderm) to already (iv) differentiated tissue-committed (monopotent) populations of stem cells (Table 1).

According to the definition a PSC is a stem cell that is able to give rise to all cells present in the embryo during development. Therefore PSC contribute in *in vitro* cultures and *in vivo* after injection into the developing blastocyst (blastocyst complementation assay) to cells from all three germ layers, mesoderm, ectoderm and endoderm, but not to the trophoblast. The presence of PSC is very well documented during embryogenesis. The question remains if PSC exist in adult organisms and if so, are these primitive stem cells, functional in adult life?

Recently several groups reported the presence of Oct-4$^+$ cells in bone marrow (BM),[1–3] cord blood (CB),[4–7] epidermis,[8,9] heart (Mendez-Ferrer S, Prat, S, Lukic A, Diego A, Badimon JJ, Fuster, V, Nadal-Ginard, B. ES-like cells in the adult murine heart. Fourth *ISSCR Annual Meeting, 2006*), pancreas,[10] testis[11,12] and bronchial epithelium.[13] Oct-4 is an embryonic transcription factor that plays a determinant role in specification of mouse PSC in the inner cell mass (ICM) of a blastocyst and mouse embryos deficient in Oct-4 are unable to form mature blastocysts[14] and die around the time of implantation.[15] Oct-4 becomes downregulated during development, and the fact that Oct-4 had been identified in some rare cells present in adult tissues suggests that some embryonic stem cells (ESC) may persist into adulthood.

Several lines of evidence support the presence of Oct-4$^+$ PSC in adult BM. First, the expression of typical PSC markers such as Oct-4,[16] Nanog[17] (another transcription factor expressed in the developing blastocyst) and stage-specific embryonic antigen (SSEA) (surface marker of early ESC),[18] was reported at the protein and/or mRNA level in BM-derived stem cells isolated using various strategies. Accordingly, these embryonic markers were demonstrated in very small embryonic-like (VSEL)[1,7] multipotent adult progenitor cells (MAPC),[19] mesenchymal stem cells (MSC)[20] (in particular the so-called serum deprived (SD) fraction)[21] and marrow-isolated adult mulitlineage inducible (MIAMI) cells.[22] Second, the existence of PSC in hematopoietic tissues is somehow supported by 'plasticity' experiments demonstrating a robust contribution of for example BM- or CB-derived cells to regeneration of multiple non-hematopoietic organs and tissues.[23–28]

Nevertheless, several questions remain. First, it is important to elucidate whether these Oct-4$^+$ cells are functional in steady-state conditions or are merely a remnants from developmental embryogenesis that reside in a dormant state in the tissues. The dormant status of these cells could be the result of the fact that they are (i) located in non-physiological niches, (ii) exposed to inhibitors, (iii) deprived of some appropriate stimulatory signals and finally (iv) limited in pluripotency because of the erasure of the somatic imprint on some of the crucial somatically imprinted genes (e.g., H19 and IGF2). A proper methylation of these imprinted genes (different on maternal- and paternal-derived chromosomes) is the crucial mechanism that governs totipotency of the zygote and pluripotency of the PSC. These PSC deposited during early gastrulation could be activated after exposure to some appropriate signals that lead to epigenetic changes that affect the methylation status of their DNA and acetylation of histones – leading to, for example, a reestablish-

Correspondence: Dr MZ Ratajczak, Hoenig Endowed Chair in Cancer Biology, Professor and Director of Stem Cell Biology Program, University of Louisville, 580 S. Preston St, Baxter II, Rm. 119E, Louisville, KY 40202, Kentucky, USA.
E-mail: mzrata01@louisville.edu
Received 13 January 2007; revised 29 January 2007; accepted 31 January 2007; published online 8 March 2007

ment of the proper somatic imprint in dormant PSC (e.g., during organ/tissue injury or oncogenesis).

Second it is important to know if these rare cells identified in BM and other tissues proliferate in steady-state conditions and contribute to the renewal of the pool of other monopotent tissue-specific stem cells. To support this possibility, for example, BM-derived stem cells were demonstrated to differentiate *in vitro* cultures to cells from different germ layers.[19–28]

In this review we will present a hypothesis that the cells that express Oct-4, Nanog, SSEA-1 (mice) and SSEA-3/4 (human) identified in adult mammalian tissues are (i) a rare population of PSC, (ii) descendants from the germ lineage, (iii) are deposited during early gastrulation in the developing organs and (iv) may persists into adulthood.

Germ lineage a mother lineage of all cell lineages in the body

From the developmental and evolutionary point of view the main goal of the multicellular organism is to pass genes to the next generations and this process is orchestrated by the appropriate interplay between germ and somatic cell lines.[29,30] The germ line carries the genome (nuclear and mitochondrial DNA) from one generation to the next generation and is the only cell lineage which retains true developmental totipotency. In this context we can envision that all somatic cell lines are descendants from the germ line and help germ cells to accomplish this mission effectively (Figure 1).

To support this concept, we can envision a zygote, which derives directly during conception from the fusion of two germ cells (female oocyte and male sperm) as the most primitive totipotent germ stem cell able to form both embryo and extra-embryonal tissues (placenta). In a zygote haploid DNA derived from an oocyte is combined with haploid DNA of a male germ cell, sperm, and the zygote could be envisioned as a mother cell to the germ lineage which 'down the road' will give rise to (i) more differentiated cells from the germ lineage (to ensure transfer of genome to the next generation) and (ii) other somatic lineages that will provide the body to fulfill this mission, which will derive/ 'bud out' from the germ lineage during embryogenesis. Figure 1 presents this concept showing a circle of reproductive life, which begins with the establishment of the most primitive germ line cell (zygote), somatic lineages (meso-, ecto- and endoderm) and most important germ cells (oocytes or sperm), which ensure transfer of DNA to the next generation. In this context the germ lineage in order to pass genes to the progeny must establish an adult organism that will provide a 'vehicle' soma/body to fulfill this mission.[30–32]

Figure 2 shows that the germ potential of the zygote is retained in the (i) first blastomers, (ii) cells that are present in the center of a developing morula and subsequently in the (iii) cells from the ICM of a blastocyst. At this time of development, however some level of specification already occurs and the trophoectoderm 'buds out' from the germ line (Figure 1). Trophoectoderm will form the placenta and the remaining part of the blastocyst – ICM will give rise to the epiblast.

Epiblast stem cells (EPSC) as demonstrated experimentally are also pluripotent and retain germ lineage potential as well.[33] Shortly before the epiblast is about to give rise to all three germ layers (ectoderm, mesoderm and endoderm), the first morphologically identifiable precursors of primordial germ cells (PGC) in mice become specified approximately, 6.0–6.5 days post-coitum (d.p.c.) in the proximal part of the epiblast (Figure 2)[34,35] Thus precursors of PGC are the first population of stem cells that is specified in the embryo at the beginning of gastrulation. PGC in mice subsequently move for a short period of time first to the

Table 1 Developmental hierarchy in the SC compartment

Totipotent SC	Give rise to both embryo and placenta. The totipotent stem cell is a fertilized oocyte (zygote) or first blastomers after cleavage of zygote
Pluripotent SC	Give rise to all three germ layers of the embryo after injection into the developing blastocyst. Pluripotent stem cells are stem cells from the inner cell mass of the blastocyst (ICM) or EPSC. They could be also derived as immortalized cell lines in *in vitro* cultures from i) ICM cells (ESC) or from ii) primordial germ cells (PGC) (EG)
Multipotent SC	Give rise to one of the germ cell layers only, either ecto-, meso- or endoderm
Monopotent SC	Are tissue-committed stem cells that give rise to cells of one lineage, for example, hematopoietic stem cells, epidermal stem cells, intestinal epithelium stem cells, neural stem cells, liver stem cells or skeletal muscle stem cells

Abbreviations: EG, embryonic germ cells; EPSC, epiblast stem cells; ESC, embryonic stem cells; ICM, inner cell mass; PGC, primordial germ cells; SC, stem cell.

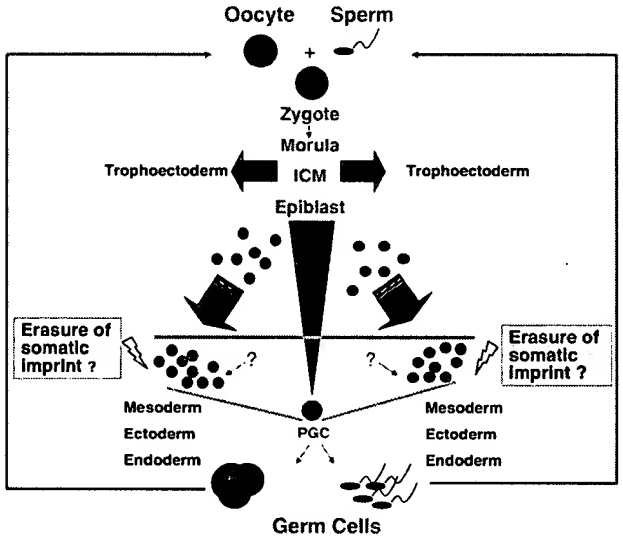


Figure 1 The cycle of life – from zygote to germ cells. From the developmental and evolutionary point of view the germ line (shown in red) carries the genome (nuclear and mitochondrial DNA) from one generation to the next and all somatic cell lines bud out (gray color) during ontogenesis from the germ line to help germ cells accomplish this mission effectively. The germ potential is established in the fertilized oocyte (zygote), and subsequently retained in the morula, ICM, EPSC, PGC and mature germ cells (oocytes and sperm). The first cells that bud out from the germ lineage are trophoectodermal cells that will give rise to the placenta. Subsequently during gastrulation EPSC are a source of PSC for all three germ layers (meso-, ecto- and endoderm) and PGC. We hypothesize that at this stage some EPSC could be deposited as Oct-4$^+$ PSC in peripheral tissues/organs (red circles). Similarly, some migrating PGC could go astray from their major migratory route to the genital ridges and become deposited as well. Furthermore, it is also possible that similarly as PGC, other EPSC deposited in the developing tissues undergo erasure of their somatic imprint (yellow arrows). This mechanism of erasure will protect developing organism from the possibility of teratoma formation. However, it will affect some of the aspects of the pluripotentiality of these cells (e.g., potential of these cells to contribute to blastocyst development).

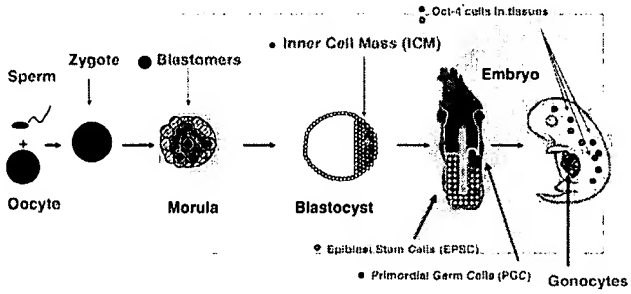


Figure 2 Retention of germ-cell potential during ontogenesis. Cells with germ potential are shown in red. The earliest and the most primitive cell in the germ line is the totipotent zygote. The germ potential is subsequently retained during development in cells located in the middle of the morula, ICM cells in the developing blastocyst, EPSC, PGC cells and germ cells in gonads. Germ potential could be also retained in rare Oct-4$^+$ EPSC deposited during development in peripheral tissues as founders for monopotent tissue committed stem cells? or those rare PGC that may go astray from their major migratory and survive in peripheral tissues (e.g., in BM or adrenal glands).

basis of alantois which is located in the extraembryonic mesoderm and then migrate into the embryo proper towards the genital ridges – where they will undergo developmental differentiation to oocytes or sperm, respectively[36] (Figure 2). Therefore one individual life could be envisioned as one link in the chain of the consecutive events aimed at the transfer of the genome by germ cells from one generation to the next. In this context, the somatic cell lines that bud from the germ line (Figure 1) merely play a supportive role.

Epiblast as a source of PSC in the developing embryo

As mentioned above PSC (Table 1) is a cell which *in vitro* at the single cell level can give rise to the cells from all three germ layers (meso-, ecto- and endoderm), however, the most valuable evidence for pluripotentiality of a stem cell is its contribution to the development of multiple organs and tissues *in vivo* after injection into the developing blastocyst. This had been very well demonstrated in the case of ESC isolated from embryos or established *in vitro* ESC lines[37–39] However, it is very difficult to get such evidence in a reproducible manner for any type of putative PSC isolated from adult tissues.

As mentioned above, EPSC are pluripotent and express SSEA-1 (mice), SSEA-3/4 (human), Oct-4 and Nanog. They will give rise to all three germ layers, ecto-, meso- and endoderm including PGC (Figure 1). Thus, the epiblast, through the process of gastrulation, is the source of all stem cells for all the germ layers and stem cells in these layers will give rise to all of the tissues and organs in the embryo. Thus EPSC could be envisioned as a founder population of PSC for multipotent stem cells for ecto-, endo- or mesoderm that give rise to unipotent stem cells that will develop given cell lineages.

We hypothesize that some pluripotent EPSC could be deposited during gastrulation in peripheral tissues (Figures 1 and 2).[30] Furthermore, there is also some additional evidence that some epiblast-derived PGC themselves during migration through the embryo proper on their way to the genital ridges might go astray and seed to peripheral tissues[40–42] Therefore, it is very likely that the cells identified in adult tissues that express ICM/epiblast/PGC markers such as SSEA-1 (mice), SSEA-3/4 (human), Oct-4 and Nanog are populations of PSC that were deposited in these tissues early during gastrulation/embryogenesis.

Regulation of pluripotency in the germ line – implications for other PSC in adult organs/tissues?

Interestingly PGC in contrast to EPSC do not reveal pluripotency. Namely, in cultures freshly isolated from embryos, PGC proliferate for a few days only, and then disappear either because they differentiate or die.[43] Furthermore, while the nuclei of migrating PGC at 8.5–9.5 d.p.c. can be successfully used as donors for nuclear transfer, nuclei from PGC at 11.5 d.p.c. and later are incompetent to support full-term development.[44] This is somehow intriguing, taking in consideration that PGC are the population of stem cells that carries 'developmental totipotency' for oocytes and sperm.

However, when PGC are cultured over murine embryonic fibroblasts and exposed *ex vivo* to three growth factors, kit ligand, leukemia inhibitory factor and basic fibroblast growth factor, they continue to proliferate and form large colonies of embryonic germ cells (EG) which similarly as ESC can be expanded indefinitely.[45–47] EG had been derived from pre- and post-migratory as well as from migratory PGC in both mice and humans and are pluripotent.[45,46] Namely, EG in contrast to PGC fully contribute to blastocyst complementation giving rise in the developing embryo to all somatic lineages and germ cells.

To explain this phenomenon at the molecular level, it is known that the pluripotency of PGC nuclei depends on the methylation status of genomic imprinted genes (e.g. H19, Igf-2, Igf-2R and Snrpn).[44,48] PGC until 9.5 d.p.c. have a somatic imprint (paternal and maternal pattern of methylation) of H19, Igf-2, Igf-2R, Snrpn – that is crucial to maintain their pluripotency. A somatic type of imprint, however, is erased by demethylation whereas these cells migrate towards the genital ridges approximately 10.5 d.p.c.[49] The erasing of the methylation (imprint) of H19, Igf-2, Igf-2R and Snrpn in early PGC could be envisioned as one of the mechanisms that shuts down PGC developmental pluripotency – and makes these cells resistant to parthenogenesis or formation of teratomas.[50,51] A proper somatic imprint is subsequently reestablished in sperm and oocytes, so that a fertilized egg expresses a developmentally proper somatic imprint of these crucial genes.

The fact that PGC-derived EG cells are pluripotent, demonstrates that the re-establishment of a proper somatic imprint is possible. We hypothesize that perhaps similar mechanism of somatic imprint erasure takes place during development not only in PGCs but also in other EPSC-derived PSCs that are deposited in the developing organs (Figure 1). It is also likely that similarly as PGC these cells under certain circumstances (e.g., tissue/organ injury) may regain a proper somatic imprint.

Presence of Oct-4$^+$ stem cells in the BM

Stem cells that express early embryonic stem cell markers including Oct-4, Nanog and SSEA-1 had been identified in BM in several potential multipotent/PSC isolated from the BM or CB such as VSELs[1,7] MSC[20,21] MAPC,[19] MIAMI,[22] unresticted somatic stem cells (USSC),[52] precursors of oocytes/spermatogonia.[53,54] It is very likely that several investigators using different isolation strategies described the same populations of stem cells but gave them different names according to the circumstance.

VSEL stem cells

The homogenous population of rare (~0.01% of BM MNC) Sca-1$^+$ lin$^-$ CD45$^-$ cells was recently purified by fluorescence-activated cell sorting (FACS) from murine BM.[1] They express (as determined by real-time quantitative PCR and immunhistochemistry) SSEA-1, Oct-4, Nanog and Rex-1 and Rif-1 telomerase protein, but do not express MHC-I and HLA-DR antigens and are CD90$^-$ CD105$^-$ and CD29$^-$. Direct electron microscopical analysis revealed that these cells display several features typical for ESC such as (i) a small size (2–4 μm in diameter), (ii) a large nucleus surrounded by a narrow rim of cytoplasm and (iii) open-type chromatin (euchromatin). Despite their small size they posses diploid DNA and contain numerous mitochondria.

Interestingly approximately 5–10% of purified VSELs if plated over a C2C12 murine myoblast cell feeder layer are able to form spheres that resemble embryoid bodies. Cells from these VSEL-derived spheres (VSEL-DS) are composed of immature cells with large nuclei containing euchromatin, and like purified VSELs are CXCR4$^+$ SSEA-1$^+$ Oct-4$^+$. Furthermore, cells from VSEL-DS, after re-plating over C2C12 cells, may again (up to 5–7 passages) grow new spheres or, if plated into cultures promoting tissue differentiation, expand into cells from all three germ cell layers. Similar spheres were also formed by VSELs isolated from murine fetal liver, spleen and thymus. Interestingly, formation of VSEL-DS was associated with a young age in mice, and no VSEL-DS were observed in cells isolated from old mice (>2 years). As VSELs express several markers of the germ cell line (fetal-type alkaline phosphatase, Oct-4, SSEA-1, CXCR4, Mvh, Stella, Fragilis, Nobox, Hdac6), they are probably closely related to a population of EPSC.

VSELs are mobile and respond robustly to an SDF-1 gradient, adhere to fibronectin and fibrinogen, and may interact with BM-derived stromal fibroblasts.[55] Confocal microscopy and time laps studies revealed that these cells attach rapidly to, migrate beneath and undergo emperipolesis in marrow-derived fibroblasts.[55] This robust interaction of VSELs with BM-derived fibroblasts has an important implication, namely that isolated BM stromal cells may be contaminated by these tiny cells from the beginning. This observation may somehow explain the unexpected 'plasticity' of marrow-derived cells with a fibroblastic morphology (e.g., MSC or MAPC). To support this, a similar population of SSEA-1$^+$ Oct-4$^+$ Nanog$^+$ Sca-1$^+$ lin$^-$ CD45$^-$ cells was also recently isolated from the BM by another team with the suggestion that these cells could be associated with MSC.[3]

MSC (multipotent mesenchymal stromal cells

MSC were initially identified as a population of BM-derived adherent bone/cartilage-forming progenitor cells.[56,57] In early passages of low-density plated MSC two morphologically distinct populations of cells were described, a population of more mature, large, flat and slowly replicating cells and one of rapidly self-renewing cells (RS cells) which are small and spindle shaped.[20] Furthermore, serum deprivation of human MSC cultures selects for expansion of so- called SD MSC.[21] These SD–MSC are small and express mRNA for embryonic markers such as Oct-4, ODC antizyme and hTERT, and proliferate more slowly than RS cells. It was postulated that these SD–MSC cells are the most primitive fraction of MSC from more mature MSC are derived. To support this further, there are recent reports that a subpopulation of undifferentiated MSC expanded from BM adherent cells could express embryonic stem cell and PSC markers such as Oct-4 and Rex1 or Oct-4 and Nanog.[58] The relationship of these undifferentiated MSC with other populations of Oct-4$^+$ cells in BM including VSELs requires further studies.

MAPC

MAPC are isolated from BM MNC as a population of CD45$^-$ GPA-A$^-$ adherent cells and they display a similar fibroblastic morphology to small MSC.[19] Thus it has been postulated that MAPC could be more primitive cells than MSC; however, the potential relationship between MSC, in particular SD–MSC cells and MAPC has yet to be established. The colonies of cells enriched in murine MAPC express SSEA-1. Recently it was reported that MAPC isolated from porcine marrow are similarly as VSELs very small, embryonic-like and express SSEA-1, Oct-4 and Nanog.[59] They are also negative for the expression of CD34, CD44, CD45, CD117 and MHC class I and class II. The growth of these rare cells depends on selected serum batches and is tightly regulated by oxygen tension. Interestingly MAPC are the only population of BM-derived stem cells that, so far as is known, contribute to all three germ layers after injection into a developing blastocyst, indicating their pluripotency.[19] The contribution of MAPC to blastocyst development, however, requires confirmation by other, independent laboratories.

MIAMI cells

This population of cells was isolated from human adult BM by culturing BM MNC in low oxygen tension conditions on fibronectin.[22] Colonies of small adherent cells express the embryonic stem cell markers Oct-4 and Rex-1 and differentiate into cells from multiple germ layers. The potential relationship of these cells to MSC and MAPC described above is not clear, although it is possible that these are overlapping populations of cells identified by slightly different isolation/expansion strategies.

BM-derived oocytes and spermatogonia

Recently somewhat unexpectedly BM was also identified as a source of oocyte-[53] and spermatogonia-like cells.[54] This observation supports to some extent the concept that during embryonic development some of the stem cells from the germ lineage (Figures 1 and 2) may go astray on their way to the genital ridges and colonize fetal liver, and subsequently by the end of the second trimester of gestation together with fetal liver-derived HSC move to the BM tissue. Accordingly, oocyte-generating germ line stem cells were found in murine BM in a set of elegant experiments in which BM transplantation restored oocyte production in normal animals sterilized by chemotherapy as well as in ataxia teleangiectasia-mutated gene-deficient mice, which are otherwise incapable of making oocytes.[53] Direct sorting analysis revealed in BM the presence of c-kit$^+$Sca-1$^-$lin$^-$ cells that expressed PGC markers such as Oct-4, Mvh, Dazl, Stella and Fragillis. Expression of all of these markers correlated with an adherent fraction of BM cells. Based on these observations the authors concluded that BM could be a potential source of germ cells that could sustain oocyte production in adulthood.

Another independent group also recently reported that BM cells may also be a source of male germ cells.[54] These cells expressed PGC markers such as Fragillis, Stella, Rnf17, Mvh and Oct-4, as well as molecular markers of spermatogonial stem cells and spermatogonia including Rbm, c-Kit, Tex18, Stra8, Piwil2, Dazl and Hsp90α. Thus BM unexpectedly is emerging as

a potential source of germ cells for reproductive medicine. So far, however, evidence is lacking that these BM-derived oocytes or spermatogonia are fully functional, capable of fertilization and can give rise to embryos.

Oct3/4$^+$ precursors of cardiomyocytes in BM

It was recently demonstrated that BM cells may form culture aggregates of Oct3/4$^+$ cells which *in vitro* cultures in the presence of platelet-derived growth factor (PDGF)-AB may differentiate into cardiomyocytes.[2] These cells were identified in BM from young and old mice, however, their potential to differentiate into cardiomyocytes by PDGF-AB-mediated paracrine/juxtacrine pathway decreased with animal age.

Oct-4$^+$ stem cells in CB

Neonatal CB is an important source of non-hematopoietic stem cells. Generally, we can envision CB as neonatal PB mobilized by the stress related to delivery. Release of several cytokines and growth factors, as well as hypoxic conditions during labor, may mobilize neonatal marrow cells into circulation.

It is well known that CB-derived cells contribute to skeletal muscle[60,61] liver[62–64] neural tissue,[65] and myocardium regeneration[66,67] and more importantly recent multiorgan engraftment and differentiation has been achieved in goats after transplantation of human CB CD34$^+$lin$^-$ cells.[68] Furthermore, several groups of investigators described the presence of Oct-4$^+$, Nanog$^+$ and SSEA-3/4$^+$ stem cells in human CB and umbilical cord matrix.[69] They were enriched by different approaches employing (i) immunomagnetic beads against CD133,[4] (ii) expansion of hematopoietic cell depleted adherent population of CB cells in the presence of thrombopoietin, kit ligand and flt3-ligand[6] or (iii) isolation of mononuclear cells by Ficoll gradient centrifugation.[5]

Recently, the small cells resembling a population of murine BM-derived VSELs was purified from CB and demonstrated at the single cell level by employing a novel two-step isolation procedure – removal of erythrocytes by hypotonic lysis combined with multiparameter FACS.[7] These CB-isolated VSELs (CB–VSEL) are very small (3–5 μm) and highly enriched in a population of CXCR4$^+$AC133$^+$CD34$^+$lin$^-$ CD45$^-$ CB mononuclear cells, possess large nuclei containing unorganized euchromatin, express nuclear embryonic transcription factors Oct-4 and Nanog and surface embryonic antigen SSEA-4. Further studies are needed to see if human CB-isolated VSELs similarly as their murine BM-derived counterparts are endowed with pluripotency. It is also not clear at this point the potential relationship of these cells to so-called CB-derived USSC. USSC are very rare cells that are detectable in approximately 40% of CB units at the frequency 1–11 cells/CB unit.[52] Unfortunately, the markers which are expressed on the founder cells for these colonies are not described. *In vitro* cultures unrestricted somatic stem cells differentiate into osteoblasts, chondroblasts, adipocytes, hematopoietic and neural cells.[52]

Oct-4$^+$ stem cells in other tissues

A population of stem cells that express markers of ESC/epiblast/PGC cells was recently described in several non-hematopoietic organs for example, in epidermis[8,9,70] bronchial epithelium,[13] myocardium (Mendez-Ferrer S, Prat, S, Lukic A, Diego A, Badimon JJ, Fuster, V, Nadal-Ginard, B ES-like cells in the adult murine heart. Fourth ISSCR Annual Meeting, 2006), pancreas,[10,71] testis[11,12] retina[72] and amniotic fluid.[73]

Oct-4$^+$ cells in bronchial epithelium

The Oct-4$^+$ long-term BrdU label-retaining cells were described at the bronchoalveolar junction of neonatal lung with the suggestion that these could be putative neonatal lung stem/progenitor cells.[13] Furthermore, lung-derived cells if cultured on collagen in serum-free conditions develop Oct-4$^+$, SSEA-1$^+$ and Sca-1$^+$ epithelial colonies with a surrounding mesenchymal stroma. These cells, presumably a subpopulation of so called Clara cells[74] could be kept for weeks in primary cultures and undergo terminal differentiation to alveolar type-2- and type-1-like pneumocytes sequentially when removed from the stroma.

Oct-4$^+$ cells in adult murine heart

By employing transgenic mice that express green fluorescence protein under a Oct-4 promoter, the presence of embryonic-like Oct-4-expressing stem cells through out the murine myocardium in particular in the atria region was demonstrated (Mendez-Ferrer S, Prat, S, Lukic A, Diego A, Badimon JJ, Fuster, V, Nadal-Ginard, B. ES-like cells in the adult murine heart. Fourth ISSCR Annual Meeting, 2006). Gene expression profile studies performed by microarrays and semiquantitative reverse transcriptase-polymerase chain reaction revealed that they express gene profiles resembling ESC. Furthermore, these cells if expanded in cultures and injected into the amniotic cavity of a developing chick embryo or a gastrulating mouse embryo confirmed wide developmental potential of these cells.

Oct-4$^+$ cells in pancreas

It was demonstrated that stem cells isolated from exocrine rat pancreas are able to differentiate in culture *in vitro* into cells from all three germ layers, have the propensity to form three-dimensional, teratoma-like structures *in vitro* and show extensive self-renewal ability and are able to expand in long-term cultures.[10] Cells isolated from these cultures express embryonic markers such as Oct-4, SSEA-1 and fetal alkaline phosphatase. Some of the clones derived from these cells were also found to differentiate into oocyte-like cells. It was shown that the expression of germ line markers such as SSEA-1, Mvh increases in these cells and in addition they acquire meiosis-specific markers such as SCP3 and DMC1.

Oct-4$^+$ stem cells in epidermis

Very small Oct-4$^+$ Nanog$^+$ stem cells distinctively different from known epithelial or melanocytic stem cells were identified in the bulge region of human hair follicles.[8] These cells if cultured *in vitro* in human embryonic stem cell medium formed so-called hair spheres that contain cells with the ability to differentiate into neurons, muscle cells, endothelial cells, adipocytes and osteoblasts. Furthermore, somehow surprisingly it was also reported that porcine fetal skin was found to be a potential source of Oct-4$^+$ germ cells that are able to differentiate into like oocyte-cumulus complexes that secreted ovarian steroid hormones and responded to gonadotropin stimulation.[70] More importantly, some of these aggregates extruded large oocyte-like cells that expressed oocyte markers. Thus these data support the concept that epidermis may contain Oct-4$^+$ stem cells endowed with germ lineage potential.

SSEA-1$^+$ cells in murine retina

A population of SSEA-1$^+$ stem cells was isolated from the murine retina.[72] These cells were highly expressed in the

developing retina, but also detectable in newborn animals and their number decreases dramatically after birth.

Oct-4$^+$ cells in amniotic fluid

A population of non-hematopoietic (CD45$^-$) SSEA$^+$ Oct-4$^+$ stem cells was recently isolated from human amniotic fluid.[73] These cells were able to differentiate into cells from all three germ layers. These data together with the observation that Oct-4$^+$ PSC could be also isolated from placental cords raises the possibility that extra-embryonic tissues could be a source of embryonic-like cells for regeneration.

Do ESC-like Oct-4$^+$ cells shuttle between BM and peripheral tissues?

Evidence accumulated that during organ damage non-hematopoietic stem cells (including VSELs) are mobilized from the BM and perhaps other tissue-specific niches into peripheral blood where they circulate in order, what we believe, to 'home' to damaged tissues and participate in tissue repair.[55] We reported that the number of these cells in peripheral blood increases also after heart infarct[75,76] and stroke.[77] Other groups found that cells expressing markers for early tissue committed stem/progenitor cells circulate during skeletal muscle[78,79] and retina epithelium damage,[80] lung injuries,[81] bone fracture,[82] ischemic damage of the kidney[83] and injury of the liver.[84] We noticed that the number of circulating non-hematopoietic stem cells expressing pluripotent/tissue committed markers (e.g., Oct-4$^+$) in peripheral blood could be increased by some mobilizing agents, for example, G-CSF.[85] in combination with compounds that block CXCR4 (e.g., T140 or AMD3100)[86,87]

Do ESC-like Oct-4$^+$ cells initiate tumor development?

In 1855 Virchow proposed the 'embryonal – rest hypothesis' of tumor formation, based on histological similarities between tumors and embryonic tissues.[88] This theory was later expanded by another pathologist Julius Conheim, who suggested that tumors develop from residual embryonic remnants 'lost' during developmental organogenesis.

The Oct-4$^+$ stem cells recently identified in adult tissues could fully support Virchow's concept. In Table 2 we proposed different scenarios how these stem cells expressing embryonic markers could contribute to tumor development.[89,90] First, if the genomic imprint in these cells is not erased they may retain post-developmental *in vivo* pluripotency and grow teratomas and teratocarciomas[91,92] Second, if they are closely related to migratory PGC, which go astray from the major migratory route to the genital ridges they may ultimately give rise to for example, germinomas and seminomas.[50,51] Third, if these cells acquire critical mutations, they may develop into the several types of pediatric sarcomas (e.g., rhabdomyosarcoma, neuroblastoma, Ewing sarcoma and Willms tumor). In support of this there is a strong correlation with the number of these Oct-4$^+$ cells which persist in postnatal tissues and the coincidence with these types of tumors in pediatric patients.[93] Finally, it is possible that these cells if mobilized at the wrong time into peripheral blood, and deposited in areas of chronic inflammation, instead of playing a role in regeneration may contribute to the development of malignancies (e.g., stomach cancer).[94]

To support this further several tumor types may express embryonic markers including Oct-4 and as reported BM-derived stem cells that may develop in the presence of cancerogens several sarcomas including teratomas.[95]

Table 2 Oct-4$^+$ cells as tumor initiating cells?

Tumor types	*Potential mechanisms*
Teratomas, teratocarcinomas	Persistent somatic imprint in Oct-4$^+$ cells (epiblast-derived?), additional mutations
Germinomas, seminomas, teratomas, dermoid cyst, hydatidiform mole	Cells left along PGC-migratory route, persistent somatic imprint?, additional mutations
Pediatric sarcomas (e.g., rhabdomyosarcoma, neuroblastoma, nephroblastoma – Wilms tumor, Ewing sarcoma)	Mutated resident or circulating Oct-4$^+$ cells in various peripheral tissue locations
Other malignancies (e.g., *Helicobacter pylori*-related stomach cancer?)	Circulating Oct-4$^+$ cells incorporated at the wrong time to the wrong place?, additional mutations

Do adult tissue-derived Oct-4$^+$ stem cells hold promise for regenerative medicine?

Humanity continually searches for the 'holy grail' to prevent sufferings caused by aging-related illnesses, and improve a quality of life in advancing age. Adult tissue-isolated Oct-4$^+$ stem cells could potentially provide a real therapeutic alternative to the controversial use of human ES cells and therapeutic cloning. Hence, while the ethical debate on the application of ES cells in therapy continues, the potential of Oct-4$^+$ stem cells is ripe for exploration. Researchers must determine whether these cells could be efficiently employed in the clinic or whether they are merely developmental remnants found in the BM and other tissues that cannot be harnessed for regeneration. The coming years will bring important answers to this question.

Acknowledgements

This work was supported by NIH Grant R01 CA106281-01 to MZR.

References

1 Kucia M, Reca R, Campbell FR, Zuba-Surma E, Majka M, Ratajczak J *et al.* A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. *Leukemia* 2006; **20**: 857–869.
2 Pallante BA, Duignan I, Okin D, Chin A, Bressan MC, Mikawa T *et al.* Bone marrow Oct3/4+ cells differentiate into cardiac myocytes via age-dependent paracrine mechanisms. *Circ Res* 2007; **100**: e1–e11.
3 Anjos-Afonso F, Bonnet D. Non-hematopoietic/endothelial SSEA-1pos cells defines the most primitive progenitors in the adult murine bone marrow mesenchymal compartment. *Blood* 2007; **109**: 1298–1306.
4 Baal N, Reisinger K, Jahr H, Bohle RM, Liang O, Munstedt K *et al.* Expression of transcription factor Oct-4 and other embryonic genes in CD133 positive cells from human umbilical cord blood. *Thromb Haemost* 2004; **92**: 767–775.
5 Zhao Y, Wang H, Mazzone T. Identification of stem cells from human umbilical cord blood with embryonic and hematopoietic characteristics. *Exp Cell Res* 2006; **312**: 2454–2464.

6 McGuckin CP, Forraz N, Baradez MO, Navran S, Zhao J, Urban R *et al.* Production of stem cells with embryonic characteristics from human umbilical cord blood. *Cell Prolif* 2005; **38**: 245–255.
7 Kucia M, Halasa M, Wysoczynski M, Baskiewicz-Masiuk M, Moldenhawer S, Zuba-Surma E *et al.* Morphological and molecular characterization of novel population of CXCR4+SSEA-4+Oct-4+ very small embryonic-like (VSEL) cells purified from human cord blood – preliminary report. *Leukemia* 2007; **21**: 297–303.
8 Yu H, Fang D, Kumar SM, Li L, Nguyen TK, Acs G *et al.* Isolation of a novel population of multipotent adult stem cells from human hair follicles. *Am J Pathol* 2006; **168**: 1879–1888.
9 Dyce PW, Zhu H, Craig J, Li J. Stem cells with multilineage potential derived from porcine skin. *Biochem Biophys Res Commun* 2004; **316**: 651–658.
10 Kruse C, Kajahn J, Petschnik AE, Maass A, Klink E, Rapoport DH *et al.* Adult pancreatic stem/progenitor cells spontaneously differentiate *in vitro* into multiple cell lineages and form teratoma-like structures. *Ann Anat* 2006; **188**: 503–517.
11 Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH *et al.* Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* 2006; **440**: 1199–1203.
12 Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H *et al.* Generation of pluripotent stem cells from neonatal mouse testis. *Cell* 2004; **119**: 1001–1012.
13 Ling TY, Kuo MD, Li CL, Yu AL, Huang YH, Wu TJ *et al.* Identification of pulmonary Oct-4+ stem/progenitor cells and demonstration of their susceptibility to SARS coronavirus (SARS-CoV) infection *in vitro*. *Proc Natl Acad Sci USA* 2006; **103**: 9530–9535.
14 Hay DC, Sutherland L, Clark J, Burdon T. Oct-4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells. *Stem Cells* 2004; **22**: 225–235.
15 Boiani M, Scholer HR. Regulatory networks in embryo-derived pluripotent stem cells. *Nat Rev Mol Cell Biol* 2005; **6**: 872–884.
16 Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* 2000; **24**: 372–376.
17 Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K *et al.* The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* 2003; **113**: 631–642.
18 Muramatsu T, Muramatsu H. Carbohydrate antigens expressed on stem cells and early embryonic cells. *Glycoconj J* 2004; **21**: 41–45.
19 Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR *et al.* Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 2002; **418**: 41–49.
20 Colter DC, Sekiya I, Prockop DJ. Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. *Proc Natl Acad Sci USA* 2001; **98**: 7841–7845.
21 Pochampally RR, Smith JR, Ylostalo J, Prockop DJ. Serum deprivation of human marrow stromal cells (hMSCs) selects for a subpopulation of early progenitor cells with enhanced expression of OCT-4 and other embryonic genes. *Blood* 2004; **103**: 1647–1652.
22 D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC. Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *J Cell Sci* 2004; **117**: 2971–2981.
23 Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR. Turning blood into brain: cells bearing neuronal antigens generated *in vivo* from bone marrow. *Science* 2000; **290**: 1779–1782.
24 Corbel SY, Lee A, Yi L, Duenas J, Brazelton TR, Blau HM *et al.* Contribution of hematopoietic stem cells to skeletal muscle. *Nat Med* 2003; **9**: 1528–1532.
25 Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R *et al.* Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* 2001; **105**: 369–377.
26 Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L *et al.* Purified hematopoietic stem cells can differentiate into hepatocytes *in vivo*. *Nat Med* 2000; **6**: 1229–1234.
27 LaBarge MA, Blau HM. Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. *Cell* 2002; **111**: 589–601.
28 Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N *et al.* Bone marrow as a potential source of hepatic oval cells. *Science* 1999; **284**: 1168–1170.
29 Weismann A. *The continuity of the germ-plasm as the foundation of a theory of heredity.* Fisher-Verlag: Jena, 1885.
30 Kucia M, Machalinski B, Ratajczak MZ. The developmental deposition of epiblast/germ cell-line derived cells in various organs as a hypothetical explanation of stem cell plasticity? *Acta Neurobiol Exp* 2006; **66**: 331–341.
31 Donovan PJ. The germ cell – the mother of all stem cells. *Int J Dev Biol* 1998; **42**: 1043–1050.
32 Zwaka TP, Thomson JA. A germ cell origin of embryonic stem cells? *Development* 2005; **132**: 227–233.
33 Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981; **292**: 154–156.
34 McLaren A. Development of primordial germ cells in the mouse. *Andrologia* 1992; **24**: 243–247.
35 McLaren A. Primordial germ cells in the mouse. *Dev Biol* 2003; **262**: 1–15.
36 Molyneaux K, Wylie C. Primordial germ cell migration. *Int J Dev Biol* 2004; **48**: 537–544.
37 Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA* 1981; **78**: 7634–7638.
38 Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA *et al.* Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol* 2000; **227**: 271–278.
39 Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Becker RA *et al.* Isolation of a primate embryonic stem cell line. *Proc Natl Acad Sci USA* 1995; **92**: 7844–7848.
40 Jordan HE. The history of the primordial germ cells in the loggerhead turtle embryo. *Proc Natl Acad Sci USA* 1917; **3**: 271–275.
41 Upadhyay S, Zamboni L. Ectopic germ cells: natural model for the study of germ cell sexual differentiation. *Proc Natl Acad Sci USA* 1982; **79**: 6584–6588.
42 Francavilla S, Zamboni L. Differentiation of mouse ectopic germinal cells in intra- and perigonadal locations. *J Exp Zool* 1985; **233**: 101–109.
43 De Felici M, McLaren A. *In vitro* culture of mouse primordial germ cells. *Exp Cell Res* 1983; **144**: 417–427.
44 Yamazaki Y, Mann MR, Lee SS, Marh J, McCarrey JR, Yanagimachi R *et al.* Reprogramming of primordial germ cells begins before migration into the genital ridge, making these cells inadequate donors for reproductive cloning. *Proc Natl Acad Sci USA* 2003; **100**: 12207–12212.
45 Matsui Y, Zsebo K, Hogan BL. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell* 1992; **70**: 841–847.
46 Shamblott MJ, Axelman J, Wang S, Bugg EM, Littlefield JW, Donovan PJ *et al.* Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc Natl Acad Sci USA* 1998; **95**: 13726–13731.
47 Turnpenny L, Brickwood S, Spalluto CM, Piper K, Cameron IT, Wilson DI *et al.* Derivation of human embryonic germ cells: an alternative source of pluripotent stem cells. *Stem Cells* 2003; **21**: 598–609.
48 Mann JR. Imprinting in the germ line. *Stem Cells* 2001; **19**: 287–294.
49 Lee J, Inoue K, Ono R, Ogonuki N, Kohda T, Kaneko-Ishino T *et al.* Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. *Development* 2002; **129**: 1807–1817.
50 Macchiarini P, Ostertag H. Uncommon primary mediastinal tumours. *Lancet Oncol* 2004; **5**: 107–118.
51 Oosterhuis JW, Looijenga LH. Testicular germ-cell tumours in a broader perspective. *Nat Rev Cancer* 2005; **5**: 210–222.
52 Kogler G, Sensken S, Airey JA, Trapp T, Muschen M, Feldhahn N *et al.* A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *J Exp Med* 2004; **200**: 123–135.
53 Johnson J, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y *et al.* Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell* 2005; **122**: 303–315.

54 Nayernia K, Lee JH, Drusenheimer N, Nolte J, Wulf G, Dressel R *et al.* Derivation of male germ cells from bone marrow stem cells. *Lab Invest* 2006; **86**: 654–663.
55 Kucia M, Reca R, Jala VR, Dawn B, Ratajczak J, Ratajczak MZ. Bone marrow as a home of heterogenous populations of nonhematopoietic stem cells. *Leukemia* 2005; **19**: 1118–1127.
56 Friedenstein AJ, Piatetzky II S, Petrakova KV. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* 1966; **16**: 381–390.
57 Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA *et al.* Precursors for fibroblasts in different populations of hematopoietic cells as detected by the *in vitro* colony assay method. *Exp Hematol* 1974; **2**: 83–92.
58 Lamoury FM, Croitoru-Lamoury J, Brew BJ. Undifferentiated mouse mesenchymal stem cells spontaneously express neural and stem cell markers Oct-4 and Rex-1. *Cytotherapy* 2006; **8**: 228–242.
59 Zeng L, Rahrmann E, Hu Q, Lund T, Sandquist L, Felten M *et al.* Multipotent adult progenitor cells from swine bone marrow. *Stem Cells* 2006; **24**: 2355–2366.
60 Gang EJ, Jeong JA, Hong SH, Hwang SH, Kim SW, Yang IH *et al.* Skeletal myogenic differentiation of mesenchymal stem cells isolated from human umbilical cord blood. *Stem Cells* 2004; **22**: 617–624.
61 Brzoska E, Grabowska I, Hoser G, Streminska W, Wasilewska D, Machaj EK *et al.* Participation of stem cells from human cord blood in skeletal muscle regeneration of SCID mice. *Exp Hematol* 2006; **34**: 1262–1270.
62 Di Campli C, Piscaglia AC, Pierelli L, Rutella S, Bonanno G, Alison MR *et al.* A human umbilical cord stem cell rescue therapy in a murine model of toxic liver injury. *Dig Liver Dis* 2004; **36**: 603–613.
63 Kakinuma S, Tanaka Y, Chinzei R, Watanabe M, Shimizu-Saito K, Hara Y *et al.* Human umbilical cord blood as a source of transplantable hepatic progenitor cells. *Stem Cells* 2003; **21**: 217–227.
64 Newsome PN, Johannessen I, Boyle S, Dalakas E, McAulay KA, Samuel K *et al.* Human cord blood-derived cells can differentiate into hepatocytes in the mouse liver with no evidence of cellular fusion. *Gastroenterology* 2003; **124**: 1891–1900.
65 Buzanska L, Machaj EK, Zablocka B, Pojda Z, Domanska-Janik K. Human cord blood-derived cells attain neuronal and glial features *in vitro*. *J Cell Sci* 2002; **115**: 2131–2138.
66 Henning RJ, Abu-Ali H, Balis JU, Morgan MB, Willing AE, Sanberg PR. Human umbilical cord blood mononuclear cells for the treatment of acute myocardial infarction. *Cell Transplant* 2004; **13**: 729–739.
67 Ma N, Stamm C, Kaminski A, Li W, Kleine HD, Muller-Hilke B *et al.* Human cord blood cells induce angiogenesis following myocardial infarction in NOD/scid-mice. *Cardiovasc Res* 2005; **66**: 45–54.
68 Zeng F, Chen MJ, Baldwin DA, Gong ZJ, Yan JB, Qian H *et al.* Multiorgan engraftment and differentiation of human cord blood CD34+ Lin- cells in goats assessed by gene expression profiling. *Proc Natl Acad Sci USA* 2006; **103**: 7801–7806.
69 Carlin R, Davis D, Weiss M, Schultz B, Troyer D. Expression of early transcription factors Oct4, Sox2 and Nanog by porcine umbilical cord (PUC) matrix cells. *Reprod Biol Endocrinol* 2006; **4**: 8.
70 Dyce PW, Wen L, Li J. *In vitro* germline potential of stem cells derived from fetal porcine skin. *Nat Cell Biol* 2006; **8**: 384–390.
71 Danner S, Kajahn J, Geismann C, Klink E, Kruse C. Derivation of oocyte-like cells from a clonal pancreatic stem cell line. *Mol Hum Reprod* 2007; **13**: 11–20.
72 Koso H, Ouchi Y, Tabata Y, Aoki Y, Satoh S, Arai K *et al.* SSEA-1 marks regionally restricted immature subpopulations of embryonic retinal progenitor cells that are regulated by the Wnt signaling pathway. *Dev Biol* 2006; **292**: 265–276.
73 De Coppi P, Bartsch Jr G, Siddiqui MM, Xu T, Santos CC, Perin L *et al.* Isolation of amniotic stem cell lines with potential for therapy. *Nat Biotechnol* 2007; **25**: 100–106.
74 Otto WR. Lung epithelial stem cells. *J Pathol* 2002; **197**: 527–535.
75 Kucia M, Dawn B, Hunt G, Guo Y, Wysoczynski M, Majka M *et al.* Cells expressing early cardiac markers reside in the bone marrow and are mobilized into the peripheral blood after myocardial infarction. *Circ Res* 2004; **95**: 1191–1199.
76 Wojakowski W, Tendera M, Michalowska A, Majka M, Kucia M, Maslankiewicz K *et al.* Mobilization of CD34/CXCR4+, CD34/CD117+, c-met+ stem cells, and mononuclear cells expressing early cardiac, muscle, and endothelial markers into peripheral blood in patients with acute myocardial infarction. *Circulation* 2004; **110**: 3213–3220.
77 Kucia M, Zhang YP, Reca R, Wysoczynski M, Machalinski B, Majka M *et al.* Cells enriched in markers of neural tissue-committed stem cells reside in the bone marrow and are mobilized into the peripheral blood following stroke. *Leukemia* 2006; **20**: 18–28.
78 Palermo AT, Labarge MA, Doyonnas R, Pomerantz J, Blau HM. Bone marrow contribution to skeletal muscle: a physiological response to stress. *Dev Biol* 2005; **279**: 336–344.
79 Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M *et al.* Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* 1999; **5**: 434–438.
80 Li Y, Reca RG, Atmaca-Sonmez P, Ratajczak MZ, Ildstad ST, Kaplan HJ *et al.* Retinal pigment epithelium damage enhances expression of chemoattractants and migration of bone marrow-derived stem cells. *Invest Ophthalmol Vis Sci* 2006; **47**: 1646–1652.
81 Gomperts BN, Belperio JA, Rao PN, Randell SH, Fishbein MC, Burdick MD *et al.* Circulating progenitor epithelial cells traffic via CXCR4/CXCL12 in response to airway injury. *J Immunol* 2006; **176**: 1916–1927.
82 Eghbali-Fatourechi GZ, Lamsam J, Fraser D, Nagel D, Riggs BL, Khosla S. Circulating osteoblast-lineage cells in humans. *N Engl J Med* 2005; **352**: 1959–1966.
83 Togel F, Isaac J, Hu Z, Weiss K, Westenfelder C. Renal SDF-1 signals mobilization and homing of CXCR4-positive cells to the kidney after ischemic injury. *Kidney Int* 2005; **67**: 1772–1784.
84 Lemoli RM, Catani L, Talarico S, Loggi E, Gramenzi A, Baccarani U *et al.* Mobilization of bone marrow-derived hematopoietic and endothelial stem cells after orthotopic liver transplantation and liver resection. *Stem Cells* 2006; **24**: 2817–2825.
85 Shyu WC, Lin SZ, Yang HI, Tzeng YS, Pang CY, Yen PS *et al.* Functional recovery of stroke rats induced by granulocyte colony-stimulating factor-stimulated stem cells. *Circulation* 2004; **110**: 1847–1854.
86 Devine SM, Flomenberg N, Vesole DH, Liesveld J, Weisdorf D, Badel K *et al.* Rapid mobilization of CD34+ cells following administration of the CXCR4 antagonist AMD3100 to patients with multiple myeloma and non-Hodgkin's lymphoma. *J Clin Oncol* 2004; **22**: 1095–1102.
87 Ratajczak MZ, Kucia M, Reca R, Majka M, Janowska-Wieczorek A, Ratajczak J. Stem cell plasticity revisited: CXCR4-positive cells expressing mRNA for early muscle, liver and neural cells 'hide out' in the bone marrow. *Leukemia* 2004; **18**: 29–40.
88 Virchow R. Editorial Archive fuer pathologische. *Anatomie und Physiologie fuer klinische Medizin* 1855; **8**: 23–54.
89 Ratajczak MZ, Kucia M, Dobrowolska H, Wanzeck J, Reca R, Ratajczak J. Emerging concept of cancer as a stem cell disorder. *CEJB* 2006; **4**: 1–15.
90 Ratajczak MZ, Zuba-Surma E, Kucia M, Reca R, Wojakowski W, Ratajczak J. The pleiotropic effects of the SDF-1-CXCR4 axis in organogenesis, regeneration and tumorigenesis. *Leukemia* 2006; **20**: 1915–1924.
91 Rossant J, Papaioannou VE. The relationship between embryonic, embryonal carcinoma and embryo-derived stem cells. *Cell Differ* 1984; **15**: 155–161.
92 Andrews PW, Matin MM, Bahrami AR, Damjanov I, Gokhale P, Draper JS. Embryonic stem (ES) cells and embryonal carcinoma (EC) cells: opposite sides of the same coin. *Biochem Soc Trans* 2005; **33**: 1526–1530.
93 Barr FG. Molecular genetics and pathogenesis of rhabdomyosarcoma. *J Pediatr Hematol Oncol* 1997; **19**: 483–491.
94 Houghton J, Stoicov C, Nomura S, Rogers AB, Carlson J, Li H *et al.* Gastric cancer originating from bone marrow-derived cells. *Science* 2004; **306**: 1568–1571.
95 Liu C, Chen Z, Chen Z, Zhang T, Lu Y. Multiple tumor types may originate from bone marrow-derived cells. *Neoplasia* 2006; **8**: 716–724.

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Biochemical and Biophysical Research Communications 341 (2006) 291–298
BBRC
www.elsevier.com/locate/ybbrc

Expansion and redifferentiation of adult human pancreatic islet cells

Limor Ouziel-Yahalom [a], Michal Zalzman [a], Leeat Anker-Kitai [a], Sarah Knoller [a], Yael Bar [a], Mariela Glandt [b], Kevan Herold [b], Shimon Efrat [a,*]

[a] *Department of Human Genetics and Molecular Medicine, Sackler School of Medicine, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel*
[b] *Naomi Berrie Diabetes Center, Division of Endocrinology, Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA*

Received 29 November 2005
Available online 19 January 2006

Abstract

β-cell replacement represents the ultimate cure for type 1 diabetes, however it is limited by availability of organ donors. Adult human islets are difficult to propagate in culture, and efforts to expand them result in dedifferentiation. Here we describe conditions for expansion of adult human islet cells, as well as a way for their redifferentiation. Most cells in islets isolated from human pancreata were induced to replicate within the first week of culture in expansion medium. Cells were propagated for 16 population doublings, without a change in replication rate or noticeable cell mortality, representing an expansion of over 65,000-fold. Replication was accompanied by a decrease in expression of key β-cell genes. Shift of the cells to differentiation medium containing betacellulin resulted in redifferentiation, as manifested by restoration of β-cell gene expression and insulin content. These methods may allow transplantation of functional islet cells from single donors into multiple recipients.

Keywords: Pancreatic beta cells; Betacellulin; Cell replication; Dedifferentiation; Insulin production

Type 1 diabetes is caused by autoimmune destruction of the pancreatic islet insulin-producing β cells. Insulin administration does not prevent long-term complications of the disease, since the optimal insulin dosage is difficult to adjust. Replacement of the damaged cells with regulated insulin-producing cells is considered the ultimate cure for type 1 diabetes. Transplantation of human intact pancreas or isolated islets has been severely limited by the scarcity of human tissue donors, leading to extensive efforts to develop an abundant in vitro source of human insulin-producing cells. Isolated human islets have been difficult to expand in tissue culture without partial or complete loss of function [1–3]. Development of differentiated islets from pancreatic duct cells has been demonstrated in cell cultures of human [4,5] and mouse [6] pancreatic cells, and the occurrence of pluripotent cells in human islets [7] and adult mouse pancreas [8] has been reported. However, the expansion capacity of these cells in vitro was shown to be quite limited. In contrast, a recent report based on cell-lineage tracing suggested that islet insulin-expressing cells maintain a renewal capacity and constitute the major source for islet regeneration following injury [9]. We sought to develop culture conditions which allow significant expansion of adult human islets from cadaver donors, as well as treatments to restore their differentiation following expansion.

Methods

Islet isolation. Human islets were isolated essentially as described [10]. Briefly, islets were isolated from pancreata obtained from organ donors after a cold ischemia time of 6.1 ± 2.9 h. The pancreatic duct was perfused with a cold enzyme mixture containing Liberase HI (Roche, Indianapolis, IN). Tissue was then transferred to a Ricordi chamber and separated by gentle mechanical agitation and enzymatic digestion at 37 °C. Islets were purified with the use of discontinuous gradients of Ficoll-diatrizoic acid [11] in an aphaeresis system (model2991, Cobe Laboratories, Lakewood, CO). The discontinuous Ficoll gradient used solution densities of 1.108, 1.096, and 1.037 g/ml layered upon each other before the separation step. During centrifugation, islets migrated to the interfaces between 1.037 and

* Corresponding author. Fax: +972 3 640 9950.
E-mail address: sefrat@post.tau.ac.il (S. Efrat).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.12.187

1.096 g/ml, and 1.096 and 1.108 g/ml [12]. The cellular material between these two interfaces was pooled and had a final purity of 71.5 ± 26.2% and a viability of 85.2±6.7%. The stimulation index (insulin secreted in response to 16.7/1.67 mM glucose) was 3.27 ± 1.78. Islets were maintained in CMRL 1066 medium at 37 °C with 5% CO_2 for 1–7 days before use.

Cell culture. Isolated islets were trypsinized and cultured in CMRL 1066 medium containing 5.6 mM glucose and supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml amphotericin B (expansion medium). The cultures were refed twice a week and split 1:2 once a week. For incubation in serum-free medium, the cells were placed in CMRL 1066 medium containing antibiotics, in the presence of 10 μg/ml insulin, 5.5 μg/ml transferrin, and 5 ng/ml selenium (ITS, Sigma–Aldrich, Steinheim, Germany). The following factors were added to the media for 6 days: activin A (Cytolab/PreproTech Asia, Rehovot, Israel; 10 nM), betacellulin (BTC; R&D Systems, Minneapolis, MN, at the indicated concentrations), and exendin-4 (Sigma–Aldrich; 10 nM).

Histological analyses. Cells were plated in 24-well plates on sterilized coverslips and fixed in 4% paraformaldehyde. For Ki67 staining cells were permeabilized with 0.25% NP40 for 10 min. Cells were blocked for 10 min at room temperature in 1% bovine serum albumin, 10% FBS, and 0.2% saponin, and incubated with the following primary antibodies diluted in blocking solution, overnight at 4 °C: mouse-anti-insulin 1:1000 (Sigma, St. Louis, MI); rabbit-anti-Ki67 1:50 (ZYMED Laboratories Inc., South San Francisco, CA); rabbit-anti-PC1/3 1:500; rabbit-anti-PC2 1:1000 (both gifts from D. Steiner); rabbit-anti-PP 1:200 (DakoCytomation, Denmark); rabbit-anti-Pdx1 1:10,000 (a gift of C. White); and mouse-anti-vimentin 1:400 (Sigma, St. Louis, MI). The bound antibody was visualized with a fluorescent secondary antibody: Cy3-goat-anti-mouse and -anti-rabbit 1:200 (Biomeda, Foster City, CA); and Alexa Fluor 488-goat-anti-rabbit 1:200 (Molecular Probes Europe BV, Leiden, The Netherlands), under a Zeiss confocal microscope. The specificity of the primary antibodies was demonstrated using COS7 cells (data not shown). Nuclei were visualized with DAPI (Roche) staining for 5 min at room temperature. BrdU staining was performed following a 24-h labeling period as previously described [13]. Nuclear area was quantitated with ImageJ software (NIH, version 1.33u) by counting cells in >4 random fields.

RNA analyses. Total RNA was extracted using High Pure RNA isolation kit (Roche Molecular Biochemicals, Mannheim, Germany). Specific transcripts were analyzed with SuperScript III (Invitrogen Life Technologies, Carlsbad, CA) RT-PCR kit according to the manufacturer. cDNA was amplified for 30–40 cycles (94 °C for 45 s; annealing for 45 s; 72 °C for 40 s), using the primer pairs and annealing temperatures listed in Supplementary Table 1 online. PCR products were separated by electrophoresis in 1.5% agarose gels and visualized by ethidium bromide staining. Real-time cDNA quantitation was performed using Assays-on-Demand kits and an ABI Prism 7000 Sequence Detector (both from Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. All reactions were done in triplicate. The results were normalized to human large ribosomal protein P0 cDNA.

Insulin and human C-peptide secretion and cell content. Insulin secretion was measured by static incubation as previously described [14]. Cells were plated in 24-well plates at 5×10^4 cells per well. The cells were preincubated for 1 h in Krebs–Ringer buffer (KRB), followed by incubation for 30 min in KRB containing 0.5 mM 1-isobutyl 3-methylxanthine (IBMX) and glucose at the indicated concentrations. The cells were then extracted in acetic acid, and the amount of insulin in the buffer and cell extract was determined using an ELISA kit (Mercodia, Uppsala, Sweden), which recognizes only mature insulin. Human C-peptide in the buffer and cell extract was determined using an ELISA kit (Mercodia, Uppsala, Sweden) according to the manufacturer.

Statistical analyses. Significance was determined using a two-tailed Student's *t* test.

Results

Islet preparations devoid of detectable ductal sheets (Fig. 1) were trypsinized and cultured in CMRL 1066 medium containing 5.6 mM glucose and supplemented with 10% FBS. Under these conditions the vast majority of cells adhered to the plate and formed a monolayer of cells with epithelial morphology. Cell size in the primary culture varied about 10-fold, as judged by measurement of nuclear area. Up to 60% of the islet cells were induced to replicate within the first 10 days of culture, as judged by BrdU incorporation (Table 1). After 3 days in culture, 11.4% of the cells replicated. Two days later the percent of replicating cells increased to 41.5%, and by 10 days in culture (3 days after the first passage) the percentage rose to 59.5%. In sections of normal pancreas about 80% of islet cells stain for insulin. However, in isolated human islets the percent of insulin$^+$ cells is lower, reflecting a rapid dedifferentiation. In the dissociated islet culture, intense insulin staining was visible after 3 days in culture

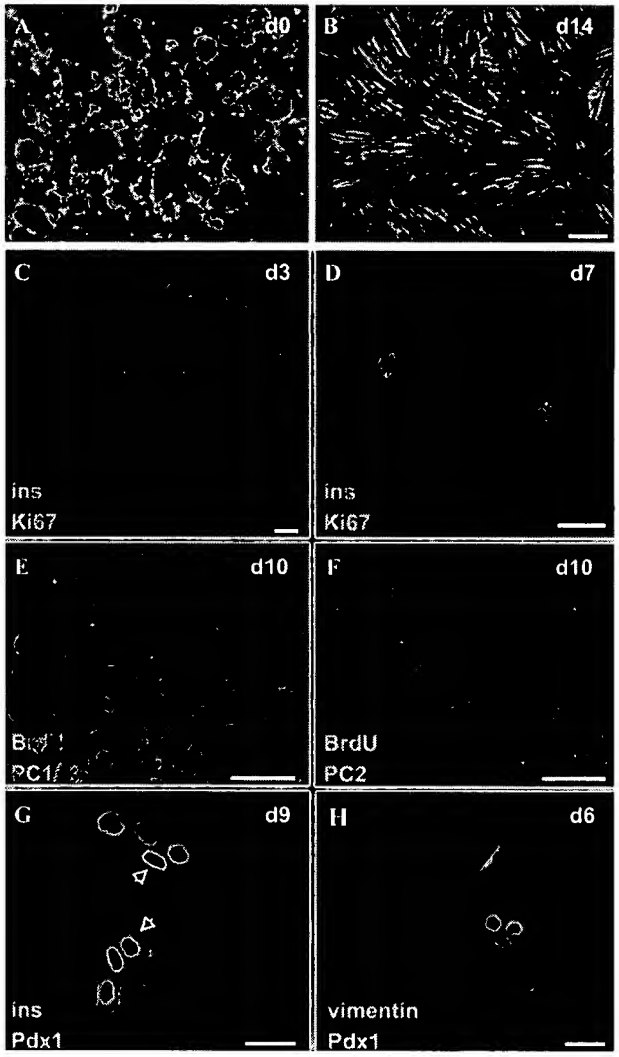


Fig. 1. Establishment of PHID cells in tissue culture. Immunofluorescence analyses following the indicated number of days during the first 2 weeks in culture. The top two panels are phase contrast images of intact islets (A) and cell monolayer (B). All nuclei in (C and E–G) are stained blue with DAPI. The arrows in (G) point to ins-negative, Pdx1-positive cells. Bar = 50 μm, except for (D, G, and H), in which bar = 20 μm.

Table 1
Effect of time in culture and BTC treatment on cell size, replication, and insulin expression

Time in culture	N[a]	% replicating	% insulin$^+$	Nuclear area (μm^2)		
				All cells	Replicating	Insulin$^+$
3 days	2481	11.4	15.4[c]	89 ± 51[b,c]	142 ± 59[b,c]	57 ± 26[d]
5 days	1787	41.5	9.4[c]	143 ± 67[c]	175 ± 60[b,c]	50 ± 35[d]
10 days	2732	59.5	5.6[c]	139 ± 47[b]	142 ± 52[c]	35 ± 18[d]
5 weeks	1263	53.5	0.5[c]	214 ± 97[b,c]	171 ± 65	n.d.
5 weeks + 6 days BTC	1954	25.1	10.9[c]	199 ± 118	174 ± 105[c]	43 ± 27[d]

[a] Number of individual nuclei analyzed.
[b] p values compared with the immediate group below in the same column <4.5E−9.
[c] p values compared with the group immediately to the right <1.3E−7.
[d] p values compared with all cells on the same day <1.84E−33.
[e] Intensely stained cells.

in 15.4% of the cells. Insulin staining was restricted to the smallest cells (nuclear area 57 ± 26 μm^2). Most of these cells were not labeled with two markers of proliferating cells, BrdU and Ki67 (Fig. 1). Nevertheless, occasional Ki67$^+$ cells manifesting weak insulin staining could be observed (Fig. 1). In contrast, BrdU staining was seen in the majority of the larger (nuclear area 142 ± 59 μm^2), insulin-negative cells. After 5 days in culture, the average cell size increased by 60% (nuclear area 143 ± 67), and the percent of insulin$^+$ cells decreased to 9.4%. After 10 days in culture the average cell size was unchanged and the percent of insulin$^+$ cells was only 5.6%. Nevertheless, most of the replicating cells stained for the β-cell markers prohormone convertase (PC) 1/3 and PC2 (Fig. 1). The loss of insulin expression was faster than that of Pdx1, as seen in Fig. 1G (arrows), showing ins-negative cells stained for Pdx1. Expression of the mesenchymal marker vimentin was observed in all the cells following 6 days in culture, including in cells that still expressed Pdx1 (Fig. 1H) and insulin (data not shown).

The doubling time of this cell population was 7 days. No significant cell mortality was observed, as judged by an apoptosis assay (data not shown). These cells, termed PHID (proliferating human islet-derived) cells, were continuously propagated for 16 passages without a noticeable change in cell replication rate and without detectable apoptosis. This passage number represents an expansion of over 65,000-fold. After 4 months of continuous replication, significant cell senescence developed, as manifested by a greatly reduced replication rate, about once in 2 weeks. These results were reproducible with islets from multiple donors, both males and females, aged 27–73.

Cell replication was accompanied by a decrease in transcription of key genes expressed in normal, quiescent beta cells. Thus, Insulin mRNA levels decreased considerably by passage 3 and were barely detectable thereafter (Fig. 2A). Similarly, expression of the β-cell transcription factors Pdx1, Beta2, and Nkx2.2, as well as the glucose transporter GLUT2, was also downregulated. In contrast, reduced levels of transcripts encoding the transcription factor Isl1, as well as PC1/3 and glucokinase (GK), persisted

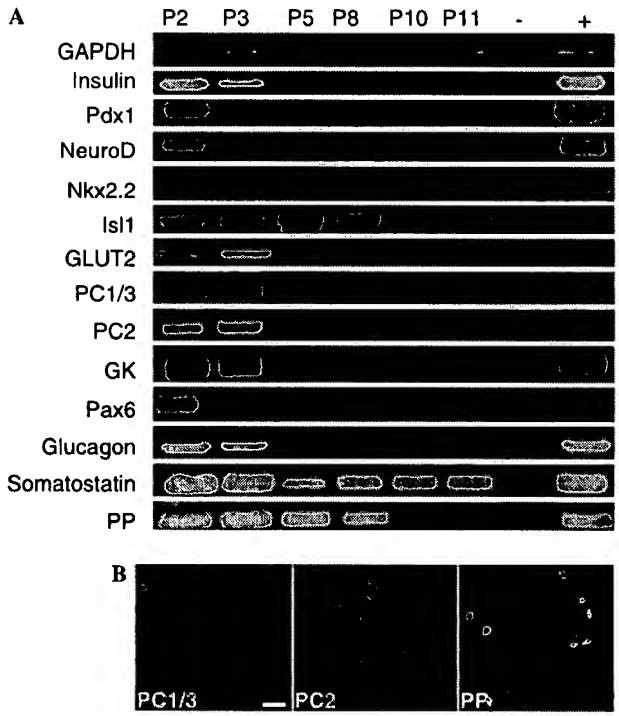


Fig. 2. Dedifferentiation of PHID cells. (A) RT-PCR analysis of gene expression in PHID cells following the indicated number of passages. Transcripts were analyzed with the indicated primers, in comparison with a negative control (−, minus-template) and a positive control (+, human islet RNA). The GAPDH gene was used to monitor mRNA and cDNA quality and loading. (B) Immunofluorescence analyses of cells at p.5. Bar = 50 μm.

in the proliferating cells. Similar to insulin, glucagon expression, as well as expression of the α-cell transcription factor Pax6, also decreased between passages 2 and 5. In contrast, expression of the genes encoding the islet hormones pancreatic polypeptide (PP) and somatostatin persisted until later passages. PC1/3 and PC2 immunostaining was detectable in the majority of cells at p.5 (Fig. 2B), indicating that the proliferating population was derived from β cells and continued to express β-cell markers. In addition, PP staining was observed in a large percent

Table 2
Gene expression in dedifferentiated PHID cells at p.5

Gene	Relative expression
Insulin	0.01 ± 0.01
NKX2.2	0.002 ± 0.00
GK	0.13 ± 0.03
PC1/3	0.11 ± 0.01
PC2	0.13 ± 0.01

RNA levels in PHID cells were quantitated by real-time RT-PCR and expressed as a fraction of the levels in freshly isolated human islets. Values are means ± SD ($n = 3$).

of the cells (Fig. 2B). These findings were confirmed by real-time RT-PCR quantitation (Table 2).

The percent of proliferating cells remained stable following 5 weeks in culture, while the percent of insulin$^+$-cells decreased to 0.5% (Table 1). The average nuclear area, compared with that of the 3-day culture, increased by that time 2.4-fold (Table 1).

To induce redifferentiation of the dedifferentiated PHID cells, they were treated with a number of factors reported to affect β-cell differentiation and proliferation. As seen in Fig. 3A, treatment with BTC resulted in restoration of expression of insulin mRNA, while exendin-4 had only a small effect at the concentration tested. In contrast, shift of the cells to serum-free medium for 6 days, or the use of activin A, did not restore insulin expression. The effect of BTC was further evaluated. A 6-day treatment with BTC restored expression of multiple β-cell genes, including insulin, transcription factors (Pdx1, NeuroD, Nkx2.2, and Nkx6.1), a gene involved in glucose sensing (GLUT2), and the β-cell marker islet amyloid polypeptide (IAPP) (Fig. 3B). In addition, expression of Pax6 and glucagon was also increased. Intense insulin immunofluorescence was visible in 10.9% of cells following treatment with BTC, a 22-fold increase (Fig. 4C, Table 1). The intense insulin staining was restricted to the smallest cells (Fig. 4C, Table 1), which did not label with BrdU or Ki67. A similar percent of cells manifested intense staining for human C-peptide (Fig. 4D). In addition to the intense insulin staining, a weak staining for insulin was visible in many replicating cells following treatment with BTC (Fig. 4B). The percent of replicating cells decreased from 53.5% to 25.1% following BTC treatment (Table 1).

Consistent with re-appearance of insulin mRNA and immunostaining, insulin content increased in cells treated with BTC (Tables 3 and 4). A considerable difference was observed between the 9 donors tested with respect to the maximal insulin content restored (Table 4). Variability was also noted with respect to the most effective concentration of BTC. Thus, in donor #5 BTC at 1 nM induced a 396× increase in insulin content, while in donor #7 the maximal increase of 19× was obtained with 6 nM BTC. The restored insulin content in donor #5 represents 20% of total cellular protein, a level in the range of that of normal β cells (insulin content in human pancreata varies

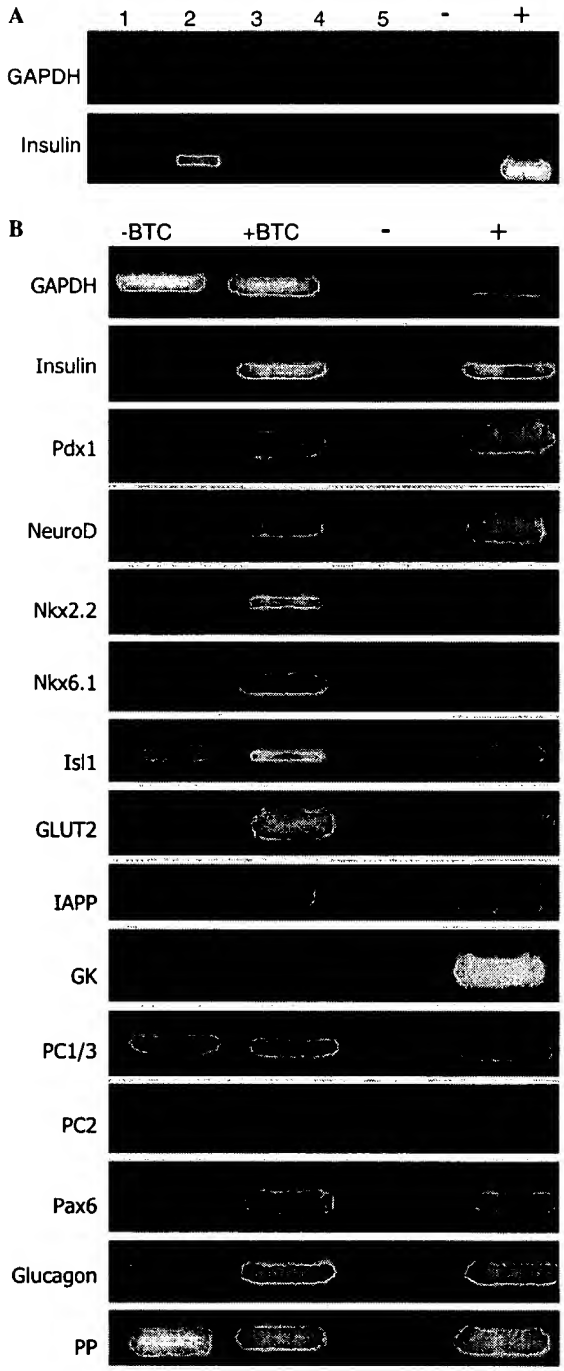


Fig. 3. Redifferentiation of PHID cells at p.5 following treatment with BTC. (A) RT-PCR analysis of cells treated with exendin-4 (lane 1), BTC (lane 2), activin A (lane 3), serum-free medium (lane 4), and expansion medium (lane 5). (B) RT-PCR analysis of cells treated with BTC. In both A and B human islet RNA was used as positive control (+), however in B its amounts were 5× lower, compared with PHID cell RNA. The negative control (−) is reaction mix without template.

widely. The study in [15] cites values between 1 and 15 mg per pancreas, which contains about 10^9 islet cells). The insulin content in cells from donor #7 is consistent with the finding that only 10.9% of the cells from this donor manifested intense insulin immunostaining (Fig. 4

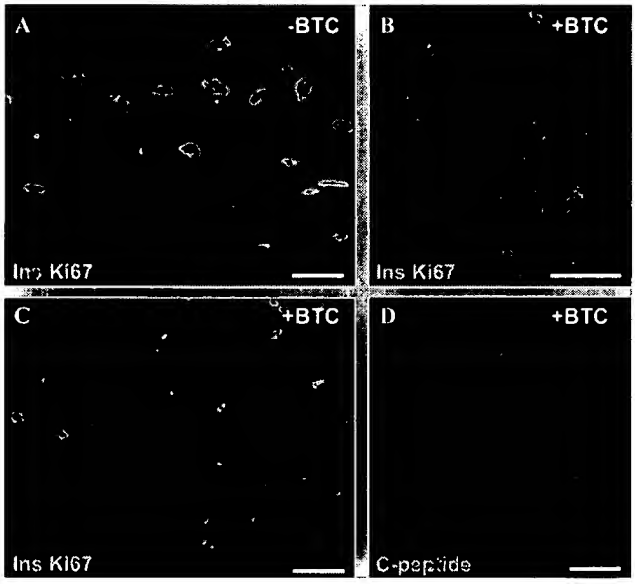


Fig. 4. Effect of BTC on insulin expression in PHID cells at p. 5. Immunofluorescence analyses of cells from donor #7 following BTC treatment. (A,B) Photographed at a high exposure for Cy3, to visualize weak insulin staining. (C,D) Photographed at a low exposure for Cy3, to visualize fields with intensely stained cells. Bar = 50 μm.

Table 3
Insulin content in PHID cells at p.5 following treatment with BTC

Donor #	BTC (nM)	Insulin content (ng/10^6 cells)	Fold increase
5	0	51 ± 1	1
	0.5	10,687 ± 138	209
	1	20,217 ± 495	396
	1.5	16,226 ± 959	318
7	0	127 ± 71	1
	0.5	1,613 ± 30	13
	1	1,751 ± 195	14
	2	1,952 ± 194	15
	4	2,020 ± 240	16
	6	2,382 ± 272	19
	8	162 ± 59	1

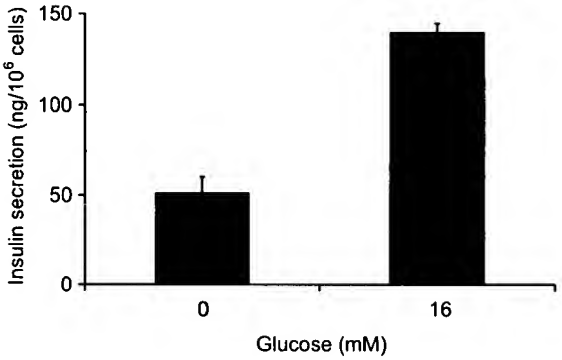


Fig. 5. Effect of BTC on insulin secretion in PHID cells at p.5. Glucose-induced insulin secretion in KRB containing 0.5 mM IBMX and the indicated concentrations of glucose during a 30-min incubation. Values are means ± SD ($n = 3$).

and Table 1). Overall, BTC showed an inductive effect in 6 out of the 9 donors tested: in 4 of them it had a pronounced effect (including in one tested only at the RNA level—see Fig. 3), while in 2 other donors it had only a 2-fold effect.

Insulin secretion from the redifferentiated cells was glucose-responsive, showing a 3-fold increase between 0 and 16 mM glucose; however, the basal level was high, compared with normal islets (Fig. 5). The percent of insulin content secreted at 16 mM glucose during a 30-min period was 0.7%, which is similar to that of normal human islets. C-peptide secretion from these cells in response to 16 mM glucose was 82 ± 21 ng/10^6 cells/30 min.

The dependence of the redifferentiated phenotype on the continuous presence of BTC was evaluated by switching the cells to regular growth medium lacking BTC. This resulted in a 38% decrease in insulin content following 7 days of culture.

Discussion

Our findings demonstrate that adult human islet cells can be significantly expanded in vitro in a simple medium.

Table 4
Effect of BTC in PHID cells derived from multiple donors

Donor #	Donor sex	Donor age	BTC effect				Serum Presence
			Insulin content (ng/10^6cells)		BTC Conc. (nM)	Fold stimulation	
			Untreated	Treated			
1	m	57	37 ± 30	66 ± 18	0.5	2	+
2	m	53	30 ± 7	575 ± 119	1	19	–
3	f	44	n.d.	n.d.	4	n.d.	–
4	f	73	129 ± 18	132 ± 9	1	0	+
5	f	51	51 ± 1	20,217 ± 495	1	396	+
6	m	49	133 ± 27	125 ± 6	1	0	+
7	f	54	127 ± 71	2,382 ± 272	6	19	+
8	f	27	126 ± 3	228 ± 8	4	2	+
9	m	58	110 ± 5	115 ± 9	4	0	+

PHID cells at passages 4–6 were treated with BTC for 6 days. The maximal increase in cellular insulin content in response to the indicated concentration of BTC is shown. Cells from donor #3 were analyzed only by RT-PCR. n.d., ELISA not done.

The cell doubling time is relatively long, about 7 days. The findings that the majority of islet cells were induced to replicate within a few days in culture (the number of replicating cells quantitated by BrdU labeling is likely an underestimate, since a BrdU pulse of 24 h may not label all cycling cells in a non-synchronous culture with a doubling time of 7 days), and that the replicating cells continued to express β-cell markers, suggest that most PHID cells originated from differentiated, insulin-expressing cells, rather than from a rare population of stem/progenitor cells which may reside in the islets, or impurities of pancreatic exocrine or duct cells present in the islet preparation. This possibility is supported by the finding of cells double-labeled for insulin and Ki67. However, a direct demonstration of this origin awaits lineage-tracing experiments. Our findings suggest that entrance into the cell cycle is accompanied by dedifferentiation and an increase in cell size. Insulin expression is rapidly lost, however, other markers of differentiated beta cells, such as PC1/3, persist in the expanded, dedifferentiated cells. In addition, expression of PP, which is restricted in normal islets to a few percent of the cells, becomes prevalent in the expanded cell population. This suggests that the dedifferentiated cells may assume the phenotype of immature cells observed during normal islet development, in which PP expression appears in a larger percent of islet cells in combination with other islet hormones [16,17]. A recent report by Gershengorn et al. [18] has shown that human islets cultured in a similar medium undergo an epithelial-to-mesenchymal transition (EMT) into cells with a doubling time of 60 h, which could be expanded 10^{12}-fold. Following serum depletion, low levels of insulin were detected in these cells. Our findings, demonstrating continuous expression of islet markers in the expanded cell population, a much slower replication rate, and a significantly higher insulin content upon redifferentiation, differ from this report. Nevertheless, expression of the mesenchymal marker vimentin was induced in all PHID cells. Its co-expression with β-cell markers (Fig. 1H) supports a partial EMT. One possible source for differences between the 2 reports is the initial culture conditions. While the study of Gershengorn et al. [18] cultured intact islets, this study cultured single cell suspension following islet dissociation by trypsinization. The culture of single cells may expedite their dedifferentiation and entrance into the cell cycle, while culture of intact islets may prolong this process and allow expansion of other, less abundant cell types.

The most significant finding reported here is the ability of BTC to restore a normal β-cell phenotype in PHID cells, as manifested by induction of expression of multiple β-cell genes, synthesis and storage of high levels of insulin, and glucose-induced insulin release. To our knowledge, the insulin content reported here is the highest for human islet cells following significant expansion in vitro. Since the differentiation protocol does not involve insulin-supplemented serum replacement components, and the cells contain insulin mRNA and C-peptide, the source of insulin is apparently biosynthesis in the PHID cells. Intense insulin staining was seen only in small, non-replicating cells, indicating that differentiation involved withdrawal from the cell cycle and a decrease in cell size. Nonetheless, a weak insulin staining was induced in many larger, replicating cells. The differentiating activity of BTC occurred in the presence of serum and was not improved by serum removal (data not shown). In addition, serum-free medium alone did not induce PHID cell differentiation. This is in contrast to a recent report using a subgroup of adherent islet cells expanded in the presence of epidermal growth factor and nerve growth factor, which showed variable effects of serum depletion on restoration of insulin expression [19]. However, the differentiated cells generated by this treatment were not glucose-responsive.

The molecular mechanism leading to BTC-induced PHID cell differentiation remains to be elucidated. BTC is a member of the epidermal growth factor family, which has been first identified in the conditioned medium of cell lines derived from mouse pancreatic β-cell tumors [20]. BTC is a potent mitogen for a number of cell types, including beta cells [21,22]. It was shown to increase islet neogenesis in alloxan- [23] and streptozotocin-treated mice [24], and accelerate islet regeneration in 90%-pancreatectomized rats [25]. In combination with activin A, BTC induced differentiation of a pancreatic exocrine cell line (AR42J) into insulin-producing cells [26]. In combination with Pdx1 expression, BTC treatment induced insulin expression in the mouse glucagonoma cell line αTC1-6 [27], and the rat intestinal cell line IEC-6 [28]. Finally, in vivo BTC treatment of mice expressing NeuroD in liver cells induced conversion of these cells into insulin-producing cells [29]. These findings suggest that BTC plays an important role in maintenance of a normal islet mass in health and disease. BTC did not manifest a mitogenic effect in PHID cells, as the percent of replicating cells decreases following BTC treatment. BTC acts by binding to members of the tyrosine kinase erbB receptor family. This triggers receptor phosphorylation and signaling to the cell nucleus through a cascade of mediators, resulting in modulation of gene transcription [30,31]. The modulated genes may include key β-cell transcription factors such as Pdx1, NeuroD, Nkx2.2, and Nkx6.1, whose expression was observed to be induced by BTC in PHID cells. In turn, these factors could stimulate insulin biosynthesis, storage, and regulated secretion. Microarray analyses of the entire spectrum of changes in gene expression affected by BTC treatment may provide further mechanistic insights.

The basis for the variability observed among donors with respect to the BTC effect remains to be elucidated. It does not appear to correlate with donor age or sex. This variability will require screening of a number of donors for finding a source of PHID cells, in which BTC can restore a sufficient insulin content to make them suitable for transplantation. Nevertheless, the properties of PHID cells following the differentiation affected by BTC render them excellent candidates for development of an abundant

source of cells, which are not genetically manipulated, for β-cell replacement therapy of type 1 diabetes. Combined with means to prevent immune rejection, this approach will allow transplantation of functional islet cells from a single donor into multiple recipients.

Acknowledgments

We thank D. Steiner and C. White for antibodies, and M. Korostishevsky for assistance with statistical analysis. The Human Islet Isolation Center at Columbia University is funded by Grants U42RR016629 and P30 DK063608 from the National Institutes of Health. This work was performed in partial fulfillment of the requirements for a Ph.D. degree of Limor Ouziel-Yahalom.

References

[1] G.M. Beattie, J.S. Rubin, M.I. Mally, T. Otonkoski, A. Hayek, Regulation of proliferation and differentiation of human fetal pancreatic islet cells by extracellular matrix, hepatocyte growth factor, and cell–cell contact, Diabetes 45 (1996) 1223–1228.

[2] T.L. Halvorsen, G.M. Beattie, A.D. Lopez, A. Hayek, F. Levine, Accelerated telomere shortening and senescence in human pancreatic islet cells stimulated to divide in vitro, J. Endocrinol. 166 (2000) 103–109.

[3] G.M. Beattie, A.M. Montgomery, A.D. Lopez, E. Hao, B. Perez, M.L. Just, J.R. Lakey, M.E. Hart, A. Hayek, A novel approach to increase human islet cell mass while preserving beta-cell function, Diabetes 51 (2002) 3435–3439.

[4] S. Bonner-Weir, M. Taneja, G.C. Weir, K. Tatarkiewicz, K.H. Song, A. Sharma, J.J. O'Neil, In vitro cultivation of human islets from expanded ductal tissue, Proc. Natl. Acad. Sci. USA 97 (2000) 7999–8004.

[5] R. Gao, J. Ustinov, M.A. Pulkkinen, K. Lundin, O. Korsgren, T. Otonkoski, Characterization of endocrine progenitor cells and critical factors for their differentiation in human adult pancreatic cell culture, Diabetes 52 (2003) 2007–2015.

[6] V.K. Ramiya, M. Maraist, K.E. Arfors, D.A. Schatz, A.B. Peck, J.G. Cornelius, Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells, Nat. Med. 6 (2000) 278–282.

[7] E.J. Abraham, C.A. Leech, J.C. Lin, H. Zulewski, J.F. Habener, Insulinotropic hormone glucagon-like peptide-1 differentiation of human pancreatic islet-derived progenitor cells into insulin-producing cells, Endocrinology 143 (2002) 3152–3161.

[8] R.M. Seaberg, S.R. Smukler, T.J. Kieffer, G. Enikolopov, Z. Asghar, M.B. Wheeler, G. Korbutt, D. Van Der Kooy, Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages, Nat. Biotechnol. 22 (2004) 1115–1124.

[9] Y. Dor, J. Brown, O.I. Martinez, D.A. Melton, Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation, Nature 429 (2004) 41–46.

[10] A.M. Shapiro, J.R. Lakey, E.A. Ryan, G.S. Korbutt, E. Toth, G.L. Warnock, N.M. Kneteman, R.V. Rajotte, Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen, N. Engl. J. Med. 343 (2000) 230–238.

[11] J.R. Lakey, G.L. Warnock, A.M. Shapiro, G.S. Korbutt, Z. Ao, N.M. Kneteman, R.V. Rajotte, Intraductal collagenase delivery into the human pancreas using syringe loading or controlled perfusion, Cell Transplant. 8 (1999) 285–292.

[12] S.P. Lake, P.D. Bassett, A. Larkins, J. Revell, K. Walczak, J. Chamberlain, G.M. Rumford, N.J. London, P.S. Veitch, P.R. Bell, et al., Large-scale purification of human islets utilizing discontinuous albumin gradient on IBM 2991 cell separator, Diabetes 38 (Suppl. 1) (1989) 143–145.

[13] I. Berkovich, S. Efrat, Inducible and reversible beta-cell autoimmunity and hyperplasia in transgenic mice expressing a conditional oncogene, Diabetes 50 (2001) 2260–2267.

[14] N. Fleischer, C. Chen, M. Surana, M. Leiser, L. Rossetti, W. Pralong, S. Efrat, Functional analysis of a conditionally-transformed pancreatic β-cell line, Diabetes 47 (1998) 1419–1425.

[15] H. Brandhorst, D. Brandhorst, M.D. Brendel, B.J. Hering, R.G. Bretzel, Assessment of intracellular insulin content during all steps of human islet isolation procedure, Cell Transplant. 7 (1998) 489–495.

[16] J. Bocian-Sobkowska, M. Zabel, W. Wozniak, J. Surdyk-Zasada, Polyhormonal aspect of the endocrine cells of the human fetal pancreas, Histochem. Cell Biol. 112 (1999) 147–153.

[17] P.L. Herrera, L. Orci, J.D. Vassalli, Two transgenic approaches to define the cell lineages in endocrine pancreas development, Mol. Cell Endocrinol. 140 (1998) 45–50.

[18] M.C. Gershengorn, A.A. Hardikar, C. Wei, E. Geras-Raaka, B. Marcus-Samuels, B.M. Raaka, Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells, Science 306 (2004) 2261–2264.

[19] A. Lechner, A.L. Nolan, R.A. Blacken, H.F. Habener, Redifferentiation of insulin-secreting cells after in vitro expansion of adult human pancreatic islet tissue, Biochem. Biophys. Res. Commun. 327 (2005) 581–588.

[20] Y. Shing, G. Christofori, D. Hanahan, Y. Ono, R. Sasada, K. Igarashi, J. Folkman, Betacellulin: a mitogen from pancreatic beta cell tumors, Science 259 (1993) 1604–1607.

[21] M.A. Huotari, J. Palgi, T. Otonkoski, Growth factor-mediated proliferation and differentiation of insulin-producing INS-1 and RINm5F cells: identification of betacellulin as a novel beta-cell mitogen, Endocrinology 139 (1998) 1494–1499.

[22] C. Demeterco, G.M. Beattie, S.A. Dib, A.D. Lopez, A. Hayek, A role for activin A and betacellulin in human fetal pancreatic cell differentiation and growth, J. Clin. Endocrinol. Metab. 85 (2000) 3892–3897.

[23] K. Yamamoto, J. Miyagawa, M. Waguri, R. Sasada, K. Igarashi, M. Li, T. Nammo, M. Moriwaki, A. Imagawa, K. Yamagata, H. Nakajima, M. Namba, Y. Tochino, T. Hanafusa, Y. Matsuzawa, Recombinant human betacellulin promotes the neogenesis of beta-cells and ameliorates glucose intolerance in mice with diabetes induced by selective alloxan perfusion, Diabetes 49 (2000) 2021–2027.

[24] L. Li, M. Seno, H. Yamada, I. Kojima, Betacellulin improves glucose metabolism by promoting conversion of intraislet precursor cells to beta-cells in streptozotocin-treated mice, Am. J. Physiol. Endocrinol. Metab. 285 (2003) E577–E583.

[25] L. Li, M. Seno, H. Yamada, I. Kojima, Promotion of beta-cell regeneration by betacellulin in ninety percent-pancreatectomized rats, Endocrinology 142 (2001) 5379–5385.

[26] H. Mashima, H. Ohnishi, K. Wakabayashi, T. Mine, J. Miyagawa, T. Hanafusa, M. Seno, H. Yamada, I. Kojima, Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells, J. Clin. Invest. 97 (1996) 1647–1654.

[27] H. Watada, Y. Kajimoto, J. Miyagawa, T. Hanafusa, K. Hamaguchi, T. Matsuoka, K. Yamamoto, Y. Matsuzawa, R. Kawamori, Y. Yamasaki, PDX-1 induces insulin and glucokinase gene expressions in alphaTC1 clone6 cells in the presence of betacellulin, Diabetes 45 (1996) 1826–1831.

[28] S. Yoshida, Y. Kajimoto, T. Yasuda, H. Watada, Y. Fujitani, H. Kosaka, T. Gotow, T. Miyatsuka, Y. Umayahara, Y. Yamasaki, M. Hori, PDX-1 induces differentiation of intestinal epithelioid IEC-6 into insulin-producing cells, Diabetes 51 (2002) 2505–2513.

[29] H. Kojima, M. Fujimiya, K. Matsumura, P. Younan, H. Imaeda, M. Maeda, L. Chan, NeuroD-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice, Nat. Med. 9 (2003) 596–603.

[30] T. Saito, S. Okada, K. Ohshima, E. Yamada, M. Sato, Y. Uehara, H. Shimizu, J.E. Pessin, M. Mori, Differential activation of epidermal growth factor (EGF) receptor downstream signaling pathways by betacellulin and EGF, Endocrinology 145 (2004) 4232–4243.

[31] C. Rescan, S. Le Bras, V.H. Lefebvre, U. Frandsen, T. Klein, M. Foschi, D.G. Pipeleers, R. Scharfmann, O.D. Madsen, H. Heimberg, EGF-induced proliferation of adult human pancreatic duct cells is mediated by the MEK/ERK cascade, Lab. Invest. 85 (2005) 65–74.

Epithelial-to-Mesenchymal Transition Generates Proliferative Human Islet Precursor Cells

Marvin C. Gershengorn,* Anandwardhan A. Hardikar, Chiju Wei, Elizabeth Geras-Raaka, Bernice Marcus-Samuels, Bruce M. Raaka

Insulin-expressing beta cells, found in pancreatic islets, are capable of generating more beta cells even in the adult. We show that fibroblast-like cells derived from adult human islets donated postmortem proliferate readily in vitro. These mesenchymal-type cells, which exhibit no hormone expression, can then be induced to differentiate into hormone-expressing islet-like cell aggregates, which reestablishes the epithelial character typical of islet cells. Immunohistochemistry, in situ hybridization, and messenger RNA measurements in single cells and cell populations establish the transition of epithelial cells within islets to mesenchymal cells in culture and then to insulin-expressing epithelial cells.

A goal of diabetes research is to generate large numbers of cells from islets of Langerhans (beta cells) for replacement therapy (*1–3*). Although beta cells proliferate in vivo (*4*) and in vitro (*5*), well-differentiated cells do not proliferate rapidly (*6*). It is likely that expansion of mature islet cells would not yield adequate cell numbers. Stem cells with potential for extensive proliferation and differentiation have been postulated but have not been identified definitively within the adult pancreas (*7–13*). Epithelial cells within the adult pancreas, however, could be isolated in vitro as undifferentiated cells and could be induced to expand and redifferentiate, thereby serving as islet precursors. Indeed, epithelial-to-mesenchymal transition (EMT) has been documented in vitro and in vivo during development and carcinogenesis (*6*, *14–16*). We show that cells from adult human islets undergo reversible EMT to produce proliferating precursors of islet-like cell aggregates (ICAs). The unexpected plasticity of human islet-derived precursor cells (hIPCs) may be exploited to generate cells for replacement therapy.

We demonstrated previously (*17*) that clonal human pancreatic cancer (PANC-1) cells and hIPCs can transition into hormone-expressing ICAs. PANC-1 cells transitioned from epithelial cells that proliferate in adherent monolayers into spherical ICAs after a change from serum-containing medium (SCM) to serum-free medium (SFM). In SCM, PANC-1 cells exhibit a "ductal" phenotype, expressing cytokeratins-7 and -19 (Ck-7/19) but no insulin or glucagon, whereas ICAs exhibit an endocrine phenotype with cells expressing insulin or glucagon. PANC-1 ICAs could be maintained in SFM for several weeks, and the phenotype could be reversed by reexposure to SCM (fig. S1A) (*18*). ICAs deaggregated rapidly with cells flattening and migrating out within 18 hours (movie S1) to recreate a population indistinguishable from parental cells by 48 hours (fig. S1A). After 12 hours in SCM, most cells that had migrated expressed Ck-7/19 and about 10% of these cells co-expressed insulin, which suggests that endocrine cells were transitioning to the ductal phenotype (movie S2). PDX1, a factor involved in insulin gene transcription (*19*), was observed primarily within ICAs.

During ICA formation in SFM, Ck-19 mRNA decreased by a factor of ~10, whereas proinsulin mRNA increased by a factor of at least 1000 from an initially undetectable level (Fig. 1A). Low levels of proglucagon transcript were detected in ductal cells and increased about 100-fold during transition. When 30-day ICAs were exposed to SFM supplemented with fetal bovine serum or epidermal growth factor (EGF) (Fig. 1A), deaggregation was associated with decreases in proinsulin and increases in Ck-19 transcript levels. When these cells were reexposed to SFM without EGF, proinsulin mRNA was up-regulated and Ck-19 mRNA was down-regulated. Furthermore, when PANC-1 ICAs were returned to SCM, some cells that migrated were positive for proinsulin mRNA and Ck-7/19 peptides (Fig. 1B), which indicated that the transition from ductal to endocrine phenotype was reversible. Transcript levels for the intermediate filament protein Ck-19 remained relatively high even when it was not detectable by immunostaining. This may be because Ck-19 stains better when in filamentous form in migratory cells rather than in nonfilamentous form in ICAs.

Transition from one epithelial phenotype to another may involve a mesenchymal intermediate (*16*). Vimentin, another intermediate filament protein, is used as a marker of mesenchymal cells (*16*). After 3 hours in SFM, PANC-1 cells migrating into ICAs (*17*) expressed vimentin in filaments, whereas parental PANC-1 cells in SCM did not (fig. S1B). Thus, PANC-1 cells appear to undergo epithelial-to-mesenchymal-to-epithelial transitions.

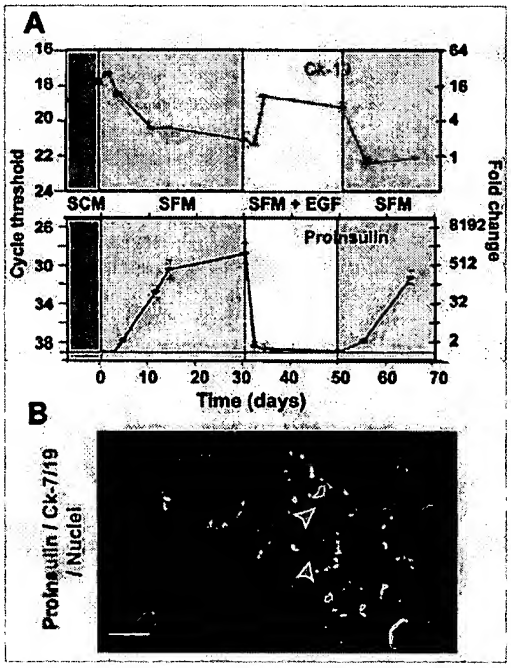


Fig. 1. PANC-1 cells undergo reversible epithelial-to-mesenchymal-to-epithelial transition. (A) Transition from "ductal" to endocrine phenotype. Cells were incubated in SCM and changed to SFM as described (*17*) on day 0, to SFM + EGF on day 30, and to SFM on day 50. Proinsulin and Ck-19 mRNAs were measured by quantitative RT-PCR. (B) Cells expressing proinsulin mRNA or Ck-7/19 peptide or both (arrowheads) were present 18 hours after ICAs were exposed to SCM. Scale bars, 10 μm.

Clinical Endocrinology Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892–8029, USA.

*To whom correspondence should be addressed. E-mail: marving@intra.niddk.nih.gov

Like PANC-1 cells, hIPCs are proliferative cells that can be induced by serum deprivation to differentiate into hormone-expressing ICAs (see below). Having observed reversible EMT in PANC-1 cells, we hypothesized that hIPCs derived from islet epithelial cells by EMT, specifically from a heterogeneous population of adherent cells that emerge from islets (fig. S2A). After 2 days in culture, more than 40% of cells were positive for C-peptide (peptide derived from the connecting region), and 3% were positive for vimentin (Fig. 2A); cells expressing both proteins were not observed. By day 7, 28% were positive for vimentin, C-peptide–positive cells decreased to 36% and, most important, 3% were positive for both proteins. The trend of increasing vimentin and decreasing C-peptide expression continued through day 14. It is noteworthy that cells positive for both proteins were not observed at the later time, which suggests that double-positive cells may reflect a transient state as C-peptide–positive cells transition to vimentin-positive hIPCs. In general, insulin-positive cells were smaller than vimentin-positive cells. Size is a characteristic of different phenotypes within a single hIPC population, because cultures of "small" cells contained cells of both sizes within 3 days (fig. S3, A and B).

In situ hybridization (*18*) showed that some adherent cells emerging from islets were positive for proinsulin mRNA and for vimentin protein (Fig. 2B), even though most cells were vimentin-negative (Fig. 2A). Vimentin staining was filamentous as in mesenchymal cells (*6*) and migratory PANC-1 cells (fig. S1B). Similarly, cells emerging from islets after 4 days express filamentous arrays of nestin, smooth muscle actin, and vimentin (fig. S2B). These data suggest that hIPCs are derived from insulin-expressing cells by EMT. To test this hypothesis, we measured proinsulin transcript in randomly selected, adherent single cells during the first 17 days of culture (Fig. 2C). During days 2 through 8, when the number of viable cells remained constant, most cells were positive

Table 1. Expression of representative epithelial and mesenchymal mRNAs in human islets and hIPCs. Total RNA was prepared from human islets within 3 days of organ donation from a single donor (Single) or pooled from 3 donors (Pooled) and from hIPCs derived from 3 individual donors (A, B, or C) at passages 8 (p8), 10 (p10), or 16 (p16). INS, proinsulin; GCG, proglucagon; GCK, glucokinase; PDX1, insulin promoter factor 1; GLP1R, glucagon-like peptide 1 receptor; CDH1, E-cadherin; CLDN3, claudin 3; CLDN4, claudin 4; OCLN, occludin; PECAM1, platelet/endothelial cell adhesion molecule (CD31); VIM, vimentin; NES, nestin; ACTA2, smooth muscle actin alpha 2; ACTG2, smooth muscle actin γ 2; ENG, endoglin (CD105); MMP2, matrix metalloproteinase 2; SNAI1, snail homolog 1; SNAI2, snail homolog 2; THY1, Thy-1 cell surface antigen; P4HA1, prolyl 4-hydroxylase alpha subunit. ND, not determined.

	qRT-PCR cycle threshold				
	Human islets		hIPCs		
	Single	Pooled	A, p8	B, p16	C, p10
Epithelial					
INS	14	14	27	>38	>38
GCG	19	20	33	35	>38
GCK	23	24	>38	>38	ND
PDX1	23	24	34	>38	ND
GLP1R	24	25	>38	>38	ND
CDH1	20	21	30	30	30
CLDN3	23	24	31	31	34
CLDN4	18	18	27	28	30
OCLN	20	21	26	26	27
PECAM1	24	24	34	31	35
Mesenchymal					
VIM	18	19	14	15	16
NES	26	27	24	24	23
ACTA2	24	25	17	18	16
ACTG2	33	31	23	21	23
ENG	25	25	21	22	22
MMP2	23	22	17	17	17
SNAI1	26	26	24	26	24
SNAI2	25	25	21	22	22
THY1	25	26	18	18	19
P4HA1	22	24	20	20	21

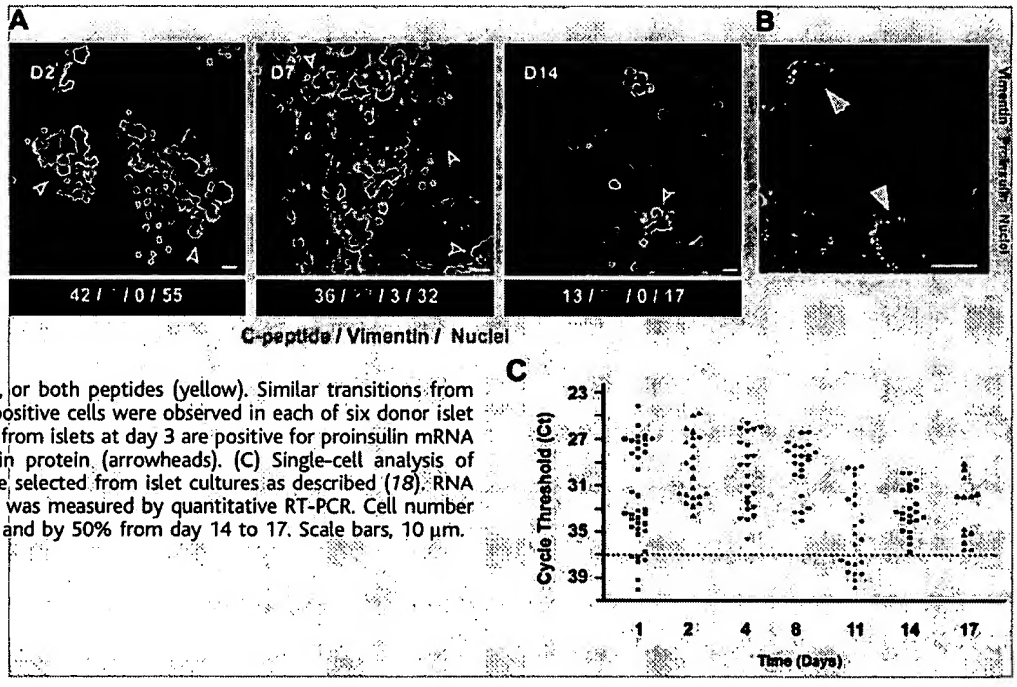


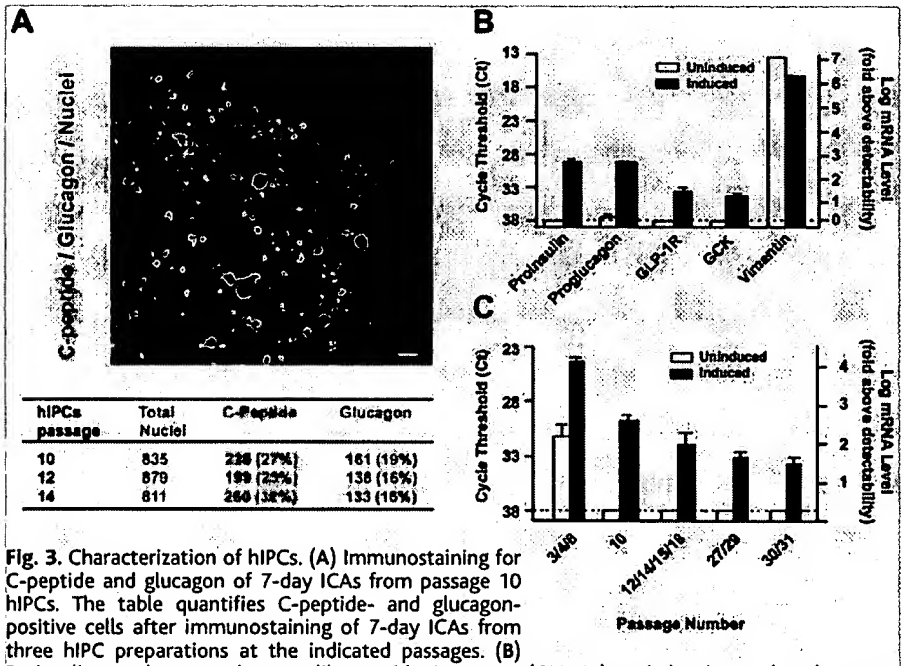
Fig. 2. hIPCs are derived from insulin-expressing cells by epithelial-to-mesenchymal transition in vitro. (A) Cells migrating out from adult human islets lose expression of C-peptide and express vimentin protein in filaments. Islet cultures were harvested with trypsin at days 2 (D2), 7 (D7), and 14 (D14); cytospun; and immunostained for C-peptide and vimentin. From >400 cells, the percentage positive for C-peptide (green), vimentin (red), both peptides (yellow), or neither (gray) is shown below each panel. Arrowheads identify cells expressing C-peptide (green), vimentin (red), or both peptides (yellow). Similar transitions from mostly negative to mostly vimentin-positive cells were observed in each of six donor islet cultures. (B) Some cells migrating out from islets at day 3 are positive for proinsulin mRNA by in situ hybridization and vimentin protein (arrowheads). (C) Single-cell analysis of proinsulin mRNA. Individual cells were selected from islet cultures as described (*18*). RNA was isolated and proinsulin transcript was measured by quantitative RT-PCR. Cell number increased twofold from day 11 to 14 and by 50% from day 14 to 17. Scale bars, 10 μm.

for proinsulin, although transcript levels were distributed over three orders of magnitude. This suggests that our culture conditions select for proinsulin mRNA-positive cells. From day 11 to 17, the level of proinsulin transcript declined in individual cells. Most important, as the cell number doubled from day 11 to 14 and increased again by one-half from day 14 to 17, the percentages of proinsulin-positive cells were 95% (day 14) and 100% (day 17). If proliferative hIPCs had arisen from proinsulin transcript-negative cells, e.g., "stem cells," a doubling of cell number would have decreased proinsulin transcript–expressing cells to 50%, and a further increase in cell number by one-half would have decreased proinsulin-expressing cells to 33%. This did not occur. In two other islet preparations, proinsulin mRNA remained detectable in 79% and 74% of cells after 14 days in culture. To demonstrate directly that insulin-expressing cells were proliferating, we labeled cells after 7 days in culture with BrdU for 40 hours and co-stained them with antibodies to BrdU and to C-peptide (fig. S4, A and B). Of the 38% of the cells in this experiment that were C-peptide–positive, more than one-fifth were also positive for BrdU, which indicated that these cells were proliferating. Taken together, our findings are most consistent with the conclusion that proliferating hIPCs originate by EMT from cells initially expressing insulin.

After 2 weeks in culture, islets had flattened to generate a monolayer of cells; residual "islets" were comprised of granular, dead cells. Harvested and reseeded cells displayed a nearly homogeneous, fibroblast-like morphology (fig. S5A). hIPCs at this stage, about 14 days after islets were placed into culture, were defined as passage 0. In 3 to 4 days, the culture reached confluence (fig. S5A). During the transition from cells within islets to hIPCs, markers for epithelial cells including E-cadherin, claudins 3 and 4, occludin, and PECAM1, as well as those specific for endocrine cells including proinsulin, proglucagon, glucokinase, PDX1, and GLP1R, decreased, whereas markers of mesenchymal cells including vimentin, nestin, smooth muscle actins α 2 and γ 2, endoglin, matrix metalloproteinase 2, snail homologs 1 and 2, Thy-1 cell surface antigen, and prolyl 4-hydroxylase increased (Table 1). Cells isolated by Beattie *et al.* (*20*) and Bouckenooghe *et al.* (*21*) appear similar to hIPCs, and they may have arisen by EMT also. Unlike many other primary cell populations derived from human or rodent islets (*10*, *20*), hIPCs exhibit substantial proliferative potential for about 90 days (doubling time of 60 hours) (fig. S5B). Cells isolated from islets by Habener and colleagues (*7*, *22*) proliferated well and may be similar to hIPCs. Cryopreserved cells resumed growth after a brief lag period at rates similar to those of cells never frozen. During the initial 3 months in culture, hIPCs expanded by almost 10^{12}. As a primary culture, hIPC proliferation slowed at later passages. hIPCs from three different islet preparations at passages 4 to 14 exhibited normal karyotypes. At early passages, hIPC populations are positive for proinsulin mRNA, but the level decreased continuously and became undetectable by passage 10 (fig. S5B). The gradual loss of proinsulin transcript may reflect the long half-life of proinsulin mRNA, estimated to be about 30 hours in rodents (*23*). Other endocrine-specific transcripts, including proglucagon, glucagon-like peptide 1 receptor, and glucokinase, also decreased and were undetectable by passage 10 (Table 1).

Up to passage 30, hIPCs could differentiate into ICAs when deprived of serum. Before differentiation, hIPCs showed immunostaining for vimentin (94% of cells), nestin (75%), and smooth muscle actin (98%) in prominent filaments (fig. S2B) like mesenchymal cells (*6*, *16*) and were negative for C-peptide. In contrast, cells within ICAs expressed C-peptide and glucagon (Fig. 3A). C-peptide staining was used to exclude detection of insulin in SFM (*24*). Immunostaining for C-peptide and glucagon of 7-day ICAs from passages 10, 12, or 14 showed that 27 ± 4% of cells stained positively for C-peptide and 17 ± 2%, for glucagon (Fig. 3A). The transition of hIPCs into ICAs increased proinsulin mRNA at least 1000-fold over initially undetectable levels and proglucagon mRNA over 100-fold (Fig. 3B). Transcripts for glucagon-like peptide 1 receptor and glucokinase also increased more than 10-fold. Thus, endocrine-specific transcripts increased when mesenchymal hIPCs transitioned into epithelial ICAs. Expression of claudin 3 and 4 mRNAs (*25*) increased whereas expression of smooth muscle actin alpha2 and gamma2 mRNAs (*16*) decreased in ICAs, further supporting the epithelial transition.

Proinsulin transcript induction was compared at different passages. At passages 3, 4, or 6, proinsulin transcript increased about 10-fold over initially detectable levels (Fig. 3C) whereas at passages 10 through 18, it increased at least 100- to 1000-fold over initially undetectable levels. At passages later than 27, smaller increases in proinsulin transcript were observed. Induction of proinsulin transcript by 100-fold or more occurred consistently in ICAs generated from mid-passage hIPCs from six separate donor islets (fig. S6). Although hIPC ICAs reproducibly exhibited marked induction of proinsulin mRNA expression, the level attained was less than 0.02% of that in human islets. Thus, hIPC ICAs are not comparable to islets in the levels of insulin (or glucagon) expression. However, cells within hIPC ICAs exhibit the following features of islets: Insulin C-peptide is detected by immunostaining (Fig. 3A); in preliminary experiments, ICAs secreted C-peptide under basal and stimulated conditions in vitro (*26*) and human C-peptide was measured in blood from three of six SCID mice implanted with ICAs under their kidney capsules, and after 14 days, implants



hIPCs passage	Total Nuclei	C-Peptide	Glucagon
10	835	228 (27%)	161 (19%)
12	879	199 (23%)	138 (16%)
14	811	260 (32%)	133 (16%)

Fig. 3. Characterization of hIPCs. (A) Immunostaining for C-peptide and glucagon of 7-day ICAs from passage 10 hIPCs. The table quantifies C-peptide- and glucagon-positive cells after immunostaining of 7-day ICAs from three hIPC preparations at the indicated passages. (B) Proinsulin, proglucagon, glucagon-like peptide-1 receptor (GLP-1R), and glucokinase (GCK) mRNAs increased, and vimentin mRNA decreased, during induction of ICA formation. (C) Induction of proinsulin mRNA at different hIPC passages. Scale bar, 20 μm.

from these three mice immunostained for human C-peptide. The blood levels of human C-peptide in the three mice were 0.22, 0.51, and 0.91 ng/ml and similar levels were found to reverse hyperglycemia in mice transplanted with insulin-expressing cells differentiated from human fetal liver progenitor cells (*27*).

In most previous attempts to generate beta cells in culture from adult islets, maintenance of insulin expression during culture was attempted (*5*, *20*, *21*). The cells obtained in these experiments did not expand well nor did they exhibit marked induction of insulin expression. Another approach was to select for cells that expressed genes, e.g., nestin, that were thought to identify precursor cells (*7*, *22*). Although the origin of these cells was not considered, they were derived from adherent islet cells and are likely similar to hIPCs, because about 75% of hIPCs are immunopositive for nestin. We show that hIPCs are "true" endocrine pancreas precursor cells that exhibit a mesenchymal phenotype before transition into epithelial clusters containing cells expressing insulin or glucagon. Indeed, hIPCs are highly proliferative and can be expanded by a factor of $>10^{12}$ and, therefore, could serve as cells for replacement therapy for diabetes if their insulin output, in particular that in response to glucose, could be optimized and they could be shown to be safe and effective upon implantation.

The origin of hIPCs is important because it provides information about the potential plasticity of insulin-expressing cells, and perhaps of other epithelial cell types, at least after culture in vitro. In contrast to the prevailing view that the source of pancreas-derived precursor cells is adult stem cells, we provide strong evidence that hIPCs are derived from insulin-expressing cells by EMT. This conclusion would be strengthened by permanently marking insulin-expressing cells in situ for cell lineage analysis as performed in mouse models (*4*, *28*), but these experiments are not possible in humans. Our studies, however, do not negate the possibility that adult stem cells are present within islets and contribute to beta cell generation in vivo.

Last, although our observations regarding EMT were made with insulin-expressing cells in vitro, a similar phenomenon may occur in vivo. Using genetically engineered mice, Dor *et al.* recently provided evidence that new insulin-expressing beta cells are derived from "preexisting" beta cells in vivo (*4*). These authors concluded, "Adult pancreatic β-cells are formed by self-duplication." Another interpretation of their data, consistent with our observations, is that new beta cells are generated from preexisting beta cells by reversible EMT.

References and Notes

1. A. M. Shapiro *et al.*, *N. Engl. J. Med.* **343**, 230 (2000).
2. B. Soria, *Differentiation* **68**, 205 (2001).
3. T. Zwillich, *Science* **289**, 531 (2000).
4. Y. Dor, J. Brown, O. I. Martinez, D. A. Melton, *Nature* **429**, 41 (2004).
5. A. Lechner, J. F. Habener, *Am. J. Physiol. Endocrinol. Metab.* **284**, E259 (2003).
6. E. D. Hay, *Acta Anat.*(Basel) **154**, 8 (1995).
7. H. Zulewski *et al.*, *Diabetes* **50**, 521 (2001).
8. V. K. Ramiya *et al.*, *Nat. Med.* **6**, 278 (2000).
9. J. G. Cornelius, V. Tchernev, K. J. Kao, A. B. Peck, *Horm. Metab. Res.* **29**, 271 (1997).
10. S. Bonner-Weir *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 7999 (2000).
11. S. Bonner-Weir, A. Sharma, *J. Pathol.* **197**, 519 (2002).
12. R. M. Seaberg *et al.*, *Nat. Biotechnol.* **22**, 1115 (2004).
13. A. Suzuki, H. Nakauchi, H. Taniguchi, *Diabetes* **53**, 2143 (2004).
14. J. P. Thiery, *Curr. Opin. Cell Biol.* **15**, 740 (2003).
15. J. P. Thiery, *Nat. Rev. Cancer* **2**, 442 (2002).
16. R. Kalluri, E. G. Neilson, *J. Clin. Invest.* **112**, 1776 (2003).
17. A. A. Hardikar, B. Marcus-Samuels, E. Geras-Raaka, B. M. Raaka, M. C. Gershengorn, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 7117 (2003).
18. Materials and methods are available as supporting material on *Science* Online.
19. H. Ohlsson, K. Karlsson, T. Edlund, *EMBO J.* **12**, 4251 (1993).
20. G. M. Beattie *et al.*, *Diabetes* **48**, 1013 (1999).
21. T. Bouckenooghe *et al.*, *Cell Transplant.* **12**, 799 (2003).
22. E. J. Abraham, C. A. Leech, J. C. Lin, H. Zulewski, J. F. Habener, *Endocrinology* **143**, 3152 (2002).
23. M. Welsh, D. A. Nielsen, A. J. MacKrell, D. F. Steiner, *J. Biol. Chem.* **260**, 13590 (1985).
24. J. Rajagopal, W. J. Anderson, S. Kume, O. I. Martinez, D. A. Melton, *Science* **299**, 363 (2003).
25. S. Tsukita, M. Furuse, *Ann. N.Y. Acad. Sci.* **915**, 129 (2000).
26. M. C. Gershengorn *et al.*, unpublished observations.
27. M. Zalzman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 7253 (2003).
28. P. L. Herrera, *Int. J. Dev. Biol.* **46**, 97 (2002).

29. We thank D. M. Harlan, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, for providing adult human cadaveric pancreatic islets.

Supporting Online Material

www.sciencemag.org/cgi/content/full/1101968/DC1
Materials and Methods
Figs. S1 to S6
References and Notes
Movies S1 and S2

25 June 2004; accepted 11 October 2004
Published online 25 November 2004;
10.1126/science.1101968
Include this information when citing this paper.

β-Arrestin 2 Regulates Zebrafish Development Through the Hedgehog Signaling Pathway

Alyson M. Wilbanks,[1,4] Gregory B. Fralish,[1,4] Margaret L. Kirby,[3] Larry S. Barak,[1,4] Yin-Xiong Li,[1,2,3]* Marc G. Caron[1,2,4]*

β-arrestins are multifunctional proteins that act as scaffolds and transducers of intracellular signals from heptahelical transmembrane-spanning receptors (7TMR). Hedgehog (Hh) signaling, which uses the putative 7TMR, Smoothened, is established as a fundamental pathway in development, and unregulated Hh signaling is associated with certain malignancies. Here, we show that the functional knockdown of β-arrestin 2 in zebrafish embryos recapitulates the many phenotypes of Hh pathway mutants. Expression of wild-type β-arrestin 2, or constitutive activation of the Hh pathway downstream of Smoothened, rescues the phenotypes caused by β-arrestin 2 deficiency. These results suggest that a functional interaction between β-arrestin 2 and Smoothened may be critical to regulate Hh signaling in zebrafish development.

Hedgehog (Hh) molecules are highly conserved morphogens that play a central role in cell proliferation and embryonic patterning (*1*, *2*). In humans, inhibitory mutations of the Sonic Hedgehog (Shh) pathway result in developmental defects such as holoprosencephaly (*3*), whereas mutations that constitutively activate the pathway lead to basal cell carcinomas (*4*) and medullablastomas (*5*). Despite extensive studies of the Hh pathway, the sequence of events leading to a biological function has yet to be fully defined. In vertebrates, extracellular Shh glycoprotein binds to the 12-transmembrane-spanning protein, Patched (Ptc), and relieves the inhibitory effect of Ptc on Smoothened (*6*). Smoothened is a signaling molecule that causes downstream uncoupling of the negative regulator Su(fu) protein from the Gli transcription factors (*7*). The subsequent nuclear translocation and DNA binding of Gli1 and Gli2, and possibly of Gli3, is followed by the increased transcription of a number of genes, including *ptc* itself (*8*) and *nkx2.2* (*9*). In contrast, the proteolytic cleavage of Gli3, promoted by cAMP-dependent protein kinase (PKA) phosphorylation in the absence of a

[1]Department of Cell Biology, [2]Department of Medicine, [3]Department of Pediatrics, [4]Howard Hughes Medical Institute Laboratories, Center for Models of Human Disease, Institute for Genome Science and Policy, Duke University Medical Center, Durham, NC 27710, USA.

*To whom correspondence should be addressed. E-mail: caron002@mc.duke.edu (M.G.C.); lyx@duke.edu (Y.-X.L.)

Curriculum Vitae

PART I:

Name: **Gordon Campbell Weir, M.D.**

Office Address: **Joslin Diabetes Center**
One Joslin Place
Boston, Massachusetts 02215

HomeAddress: **2 Larchwood Drive**
Cambridge, MA 02138

E:Mail: **gordon.weir@joslin.harvard.edu** **FAX: 617 732-2650**

Place of Birth: **Cleveland, OH**

Education:

1963 B.A.	Dartmouth College, Hanover, NH
1967 M.D.	Harvard Medical School, Boston, MA

Postdoctoral Training:

1967-1968	Intern in Medicine, University Hospitals, Cleveland, OH
1968-1970	Resident in Medicine, University Hospitals, Cleveland, OH
1970-1972	Research and Clinical Fellow in Medicine (Endocrinology and Metabolism), Massachusetts General Hospital, Boston, MA
1970-1972	Research Fellow in Medicine, Harvard Medical School, Boston, MA

Licensure and Certification:

1976	Virginia License Registration
1984	Massachusetts License Registration
1973	American Board of Internal Medicine Subspecialty-Endocrinology (Certification #034547)

Academic Appointments:

1972-1974	Instructor in Medicine, Harvard Medical School, Boston, MA
1974-1976	Assistant Professor of Medicine at the Massachusetts General Hospital, Harvard Medical School

1976-1981	Associate Professor of Medicine, Division of Endocrinology/Metabolism, Medical College of Virginia, Richmond, VA
1981-1984	Professor of Medicine, Division of Endocrinology/Metabolism, Medical College of Virginia
1983-1984	Professor of Biophysics and Physiology, Medical College of Virginia
1984-1989	Associate Professor of Medicine, Harvard Medical School
1990-	Professor of Medicine, Harvard Medical School

Hospital Appointments:

1972-1975	Assistant in Medicine, Massachusetts General Hospital, Boston, MA
1975-1976	Assistant Physician, Massachusetts General Hospital, Boston, MA
1976-1984	Attending Physician, Medical College of Virginia Hospital, Richmond, VA
1982-1984	Vice Chairman, Department of Internal Medicine, Medical College of Virginia School of Medicine, Richmond, VA
1984-1977	Active Staff, New England Deaconess Hospital, Boston
1984-1993	Chief, Section of Endocrinology, Diabetes, Metabolism, New England Deaconess Hospital, Boston, MA
1984-1993	Medical Director, Joslin Diabetes Center Boston, MA
1997-	Active Staff, Beth Israel Deaconess Medical Center, Boston

Other Professional Positions and Major Visiting Appointments:

1980-1981	Visiting Professor, University of Geneva Switzerland

Major Committee Assignments:

National and Regional:

1977	Special Review Committee for Diabetes Research and Training Center Applications, National Institutes of Health (NIAMDD)
1979-1982	Metabolism Study Section, National Institutes of Health
1979	Consultant for External Review, University of Michigan Diabetes Research and Training Center
1979-1984	Board of Directors, American Diabetes Association, Virginia Affiliate
1981-1984	Research Committee, American Diabetes Association
1983	Second National Conference on Diabetes (Reston, VA) Non-insulin Dependent Diabetes Mellitus Workshop. Insulin Secretion Workshop
1984	Consultant-Diabetes Program Mt. Sinai Medical School

1984-1987	Board of Directors, American Diabetes Association
1985-1987	Professional Education Committee, American Diabetes Association
1985-1989	ADDK-B Committee/NIADDK, National Institutes of Health, Acting Chairman, November 1987
1986-1991	Scientific Advisory Board, Organogenesis, Inc.
1987-	Scientific Advisory Board, Clinical Research Center, Brigham &Women's Hospital
1988	Research Committee, Juvenile Diabetes Foundation, Ad Hoc Member
1988-1990	International Advisory Committee, First International Congress on Pancreatic and Islet Transplantation, Stockholm
1988	International Scientific Advisory Committee (Bayer)
1989	Organizing Committee Annual American Diabetes Association Research Symposium - Marco Island, FL
1990	Organizing Committee Annual American Diabetes Association Research Symposium - Woods Hole, MA
1990-1991	American Diabetes Association, Committee on Professional Care
1991-1993	Chairman, Recognizing Diabetes Treatment Programs Task Force, American Diabetes Association
1991	Co-organizer: Satellite Conference to IDF Congress."Pancreatic beta cell 1991: Gene to Disease." Cambridge, MA
1991	Session Chairman, Third International Symposium on Islet Transplantation, Gubbio, Italy
1991-1994	National Diabetes Advisory Board, Chairman 1994
1992-1995	Research Committee, Juvenile Diabetes Foundation International
1993-	External Advisor, Yale Diabetes and Endocrinology Research Center
1994-1999	Scientific Advisory Board, Neocrin Company
1994-	Scientific Advisory Board, Carmel Biotechnology, Ltd.
1994	Satellite Conference to IDF Congress. "Pancreatic Beta Cell 1994:From Molecular Biology to Clinical Medicine. Vice-Chairman, Organizing Committee
1995-	Chairperson, Epidemiology of Diabetes Interventions and Complications (EDIC) External Advisory Committee (N.I.H.)
1996	Juror, Francqui Prize, Belgium
1997-2000	Juvenile Diabetes Foundation International Medical Science Advisory Board
1997-	Scientific Advisory Board. Diabetes Research Institute, U. Of Miami
1997-2002	Program Director, NIH/JDFI Program Project, Islet Xenotransplantation: Genetic Approaches to the Problem.
1998-2003	Associate Director, JDF Center for Islet Transplantation at Harvard Medical School
2000	Juror, Prix scientifique Joseph Maisin,, Belgium

2000	Chairperson, EASD Satellite Meeting, Eilat, Israel
2000-2002	American Diabetes Association, Scientific Sessions Committee
2000-2003	Juvenile Diabetes Research Foundation, Medical Scientific Review Committee
2002	Scientific Review Board, Steno Diabetes Center
2002	Chairman: NIH Special Study Section - review of DRTC applications
2002-2005	Steering Committee, Beta Cell Biology Consortium
2003	Co-Course Director, Joslin Symposium 2003
2004	Chair Organizing Committee - Cell Transplant Society, 7 th International Congress, Boston
2004	Ad hoc Search Committee, Chair of Medicine, Brigham and Women's
2004	Head, Diabetes Program, Harvard Stem Cell Institute .

Medical College of Virginia

1977-1984	Clinical Practice Committee, Department of Medicine
1978-1984	Board of Directors, University Internal Medicine Foundation
1979-1982	M-III Clerkship Committee, Department of Medicine
1979-1982	Endocrine Course Director (2nd yr class), School of Medicine
1981-1984	School of Medicine Promotion and Tenure Committee, Chairman 1982

Joslin Diabetes Center

1984-1993	Board of Trustees
1984-1993	Professional Advisory Council
1987-1993	Trustee and Member Affairs Committee
1989-1993	Patient Care Assessment Committee, Chairman
1994-1996	Medical Record Committee, Chairman
1996-1998	Board of Trustees

Deaconess Hospital/Beth Israel Deaconess Medical Center

1984-1993	Medical Advisory Board
1985-1993	Staff Membership Committee
1985-1989	Long Range Planning Committee
1985-1993	Executive Committee, Department of Medicine
1987-	Medical Records Committee

Harvard Medical School

1990-1993	Committee on Promotions, Reappointments and Appointments
1993-1997	Committee on Conflicts of Interest and Commitment
1996-1999	Subcommittee of Professors
1999-	Subcommittee on Xenotransplantation

Professional Societies:

1974-	American Diabetes Association
1974-1981	American Federation for Clinical Research
1976	Endocrine Society
1977-1984	Southern Society for Clinical Research
1977-1984	American Diabetes Association (Virginia Affiliate President (1981-1982). Richmond Chapter, President, 1983-1984
1978-1985	American Society for Clinical Investigation (currently emeritus member)
1979-1982	Councilor, Southern Section AFCR
1984-1990	American Diabetes Association (Massachusetts Affiliate Board of Directors)
1990-	Cell Transplant Society (Councilor 1999-2003; President-Elect 2003-4), President, 2004
1991-	International Pancreas and Islet Transplantation Association (Councilor 1999-2003)

Editorial Boards:

1976-1985	Metabolism
1980-1983	Endocrinology
1980-1982	American Journal of Physiology
1986-1988	Diabetes (Journal of the American Diabetes Association)
1987-1995	Drugs (Auckland)
1988-1994	American Journal of Physiology
1989-1991	Journal of Clinical Endocrinology and Metabolism (Editor)
1991-1994	Cell Transplantation
1992-	Diabetes, Nutrition, and Metabolism
1993-1996	Endocrinology
1997--2001	Diabetes, Editor-in-Chief
2004 -	Transplantation

Awards and Honors:

1972-1975	Daland Fellowship, American Philosophical Society
1975-1976	Capps Scholar, Harvard Medical School
1977-1981	Research Career Development Award, National Institutes of Health
1980-1981	Zyma Fellowship (Switzerland)
1980	"Best Professor" of Endocrine Course (2nd year) medical student
1986	First Kroc Lectureship, Diabetes Research Center, University of Washington
1987	Opening Lecture, Annual Belgian Diabetic Research Meeting
1988	Lecture, Research Symposium on Beta Cell Function Annual Meeting of the American Diabetes Association

1988	Soaring Pine Award, Dartmouth College Class of 1963
1988	Invited Lecturer: 13th International Diabetes Federation Congress, Sydney
1989	Invited Lecturer, Annual Meeting of the German Diabetes Association
1990	Donald M. Silver Excellence in Research Award, Juvenile Diabetes Foundation
1990	Roerig Visiting Professor in Diabetes, University of California, San Francisco
1990	John Walker Moore Visiting Professor, University of Louisville School of Medicine
1991	William Stadie Award, Philadelphia Chapter, American Diabetes Association
1991	Visiting Professor of Medicine, University of Hawaii
1991	William N. Creasy Visiting Professor in Clinical Pharmacology, State University of New York at Buffalo
1991	Eighth Annual Novo Nordisk-McGill Lecturer in diabetes mellitus, Montreal
1991	Keynote speaker, Annual Glucagon Meeting of Japan (Glucagon Kenkyukai) Osaka
1992	Louise C. Buerki Visiting Professor, Henry Ford Hospital
1993	Professor M. Ibrahim Memorial State of the Art Lecture, College of Physicians and Surgeons, Karachi, Pakistan
1994	Pfizer Visiting Professor, Jefferson Medical College
1995	Plenary lecture, German Diabetes Association
1996	Plenary lecture, Spanish Diabetes Association
1997	One of "Best Doctors in Boston", Boston Magazine
1998	Presentation. Congressional Biomedical Research Caucus on diabetes
1998	David Rumbough Award, Juvenile Diabetes Foundation
1998	Keynote speaker, Bristol Immunology Group
1998	Invited Lecture, Turkish National Diabetes Congress
1998	Presentation Congressional Diabetes Caucus (September)
1999	U. Miami/VA/Pharmacia Upjohn Visiting Professor
1999	Pfizer Visiting Professor, U. Of Rochester
1999	Invited Speaker, Scottish Diabetes Meeting
2000	Symposium Speaker, EASD, Jerusalem
2001-	Diabetes Research and Wellness Foundation Chair
2001	Littlefield Lecture, Memorial Hospital, Rhode Island
2001	Invited Lecture, Japanese Diabetes Society
2001	William G. Blackard Lecture, Medical College of Virginia, Richmond
2002	Visiting Professor - Annual Symposium, Diabetes Center, UCSF
2002	Opening Lecture - AIDPIT Study Group - Igls, Austria
2003	Symposium speaker, American Diabetes Association Annual

	Meeting
2003	Symposium speaker, IDF Meeting, Paris
2003	5th Annual Piero P. Foa Lecture, Wayne State School of Medicine
2003-2006	JDRF – The Lewis-Sebring Family Foundation Research Project
2004	Frontiers in Science Award – American Assoc. Clinical Endocrinologists
2004	Opening Lecture – The 1st Russell Berrie International Diabetes Symposium, Jerusalem
2005	Carl N. Holmes Distinguished Alumnus Award, Hawken School, Cleveland
2006	Keynote Lecture – Diabetes Technology Society Mtg. San Francisco
2006	One of the "Top Doctors in Boston" – Boston Magazine
2006	Beverly Towery Lectureship – University of Louisville
2007	Opening Lecture – American Diabetes Association Research Symposium, Stone Mountain, Georgia

PART II: Research, Teaching, and Clinical Contributions

Major Research Interests:

1. Secretion from the Islet of Langerhans

2. Insulin and glucagon secretion in non-insulin dependent diabetes

3. Transplantation of the islets of Langerhans

1A Teaching Experience:

1976-1984	2 months of attending on general medicine, yearly
1976-1984	3 months of attending on endocrinology, yearly
1976-1984	Physical Diagnosis for second year students, yearly
1976-1984	Numerous miscellaneous lectures given at all student levels and house staff
1979-1982	Course Director, second year Endocrine Block, Medical College of Virginia
1984-	Frequent attending on Endocrinology/Metabolism and Internal Medicine
1984-1993	Harvard-Joslin Course in Clinical Diabetes-CME Course Director
1986	American College of Physicians - Annual Meeting-ran workshop on complications of diabetes mellitus
1988-1990	Harvard Medical School Introduction to Clinic coordinated Brigham & Women's Introduction to diabetes at Joslin
1990	Tutor Second year students Harvard Medical School Endocrine Block

1B Teaching Awards and Results of Teaching Evaluations:

1980 "Best Professor" of second year endocrine course Medical College of Virginia

Research Trainees

Fellows		Arrival/Depart	Currrent Institution	Current Position
RN Honey, PhD	Pre-doc	1977-1980	Unknown	
DF Trent	Pre-doc	1979-1981	Virginia	Medical practice
J Brockenbrough	Pre-doc	1979-1982	Anatomy, U FL	
RM Colella	Post-doc	1982-1984	Anatomy, U Louisville	Assoc Prof
DF Fletcher	Post-doc	1979-1982	Anatomy, U East Carolina	Assoc Prof
JL Leahy	Post-doc	1982-1984	U Vermont	Prof of Medicine
Dov Gefel	Post-doc	1985-1987	Israel	Academic medicine
Grant Hendrick	Post-doc	1986-1988	Industry	Scientist
Hyun Chul Lee	Post-doc	1986-1988	Korea	Prof
George T Schuppin, PhD	Post-doc	1989-1993	Industry	Scientist
YJ Wu, MD	Post-doc	1989-1996	California	Medical practice
Eduard Montanya, MD, PhD	Post-doc	1990-1993	Hospital Bellgite, Barcelona	Prof of Medicine
Alberto Davalli, MD, PhD	Post-doc	1992-1995	Instituto Scientifico, Italy	Assoc Prof
Yoshiji Ogawa, MD	Post-doc	1992-1994	Hirosaki U, Japan	Endo & Res
Jyun-Huarg Juang, MD	Post-doc	1992-1994	Chang Gung Hospital, Taiwan	Assoc Prof Endo&Res
Raimund Weitgasser, MD	Post-doc	1993-1994	Muellener Hospital, Austin	Staff Doc&Res
Yoshihiko Noma, MD, PhD	Post-doc	1993-1994	Tokushima U, Japan	Assoc Prof-Pathology
John Capotorto, MD	Post-doc	1993-1995	State Island Hospital	Medical practice
Chung Horn Lee, MD	Post-doc	1994-1995	New Changi Hospital, Singapore	Medical practice
David H Zangen, MD	Post-doc	1994-1996	Endo, Hadassah U, Israel	Asst Prof Ped Endo
Kazuhisa Suzuki, MD	Post-doc	1994-1996	Mahoshi Hospital, Japan	Endo & Res
Nitin Trivedi, MD	Post-doc	1995-2001	Brown U, Rhode Island	Assistant Prof Medicine
Robert Quickel, MD	Post-doc	1995-1997	Wisconsin	Staff Surgeon
David Vasir, PhD	Post-doc	1995-2000	Dana Farber	Instructor
Garry Steil, PhD	Post-doc	1996-1998	Mini Med, Industry	Scientist
Krystyna Tatarkiewicz, PhD	Post-doc	1996-2001	California, Industry	Scientist
Kun-Ho Yoon, MD	Post-doc	1996-1998	St Mary's Hospital S, Korea	Prof Med/Endo&Re

				s
Jean-Christophe Jonas, MD	Post-doc	1997-1998	Belgium	Asst Prof
Maritza Garcia, MD	Post-doc	1997-1998	Mexico City Hospital	Endocrinologis t
Maria Lopez-Avalos, PhD	Post-doc	1998-2001	Universidad de Malaga	Instructor
David R Laybutt, PhD	Post-doc	1998-2001	Garvan Institute	Scientist
Mitchell Keegan, PhD	Post-doc	1998-2001	Boston, industry	Research
Abdulkadir Omer, MD	Post-doc	1998-	Joslin/Harvard	Instructor
Hideaki Kaneto, MD, PhD	Post-doc	1999-2002	Osaka University	Scientist
Valerie Duvivier, PhD	Post-doc	1999-2002	France Industry	Scientist
Yolanda Hawkins	Med Stu	2000-2001	ADA/support	
Amy A Jennings, PhD	Post-doc	2001-2001	Connecticut, industry	Scientist
Yu Bai Ahn, MD	Post-doc	2001-2003	Catholic University, Korea	Assoc Prof Med
Giovanni Patane, MD	Post-doc	2001-2002	Cattedra di Endocrinologia, Italy	
Yoshiyuki Hamamoto, MD	Post-doc	2002-2004	Hospital, Japan	Staff Doctor
Aileen King, PhD	Post-doc	2002-2005	Kings College London, UK	Scientist
Lorella Marselli, MD, PhD	Post-doc	2002-	Joslin	
Ludivina Robles, PhD	Post-doc	2003-2005	Hospital, Mexico	Staff Doctor
Tomoyuki Akashi, MD	Post-doc	2003-2006	Hospital, Japan	Staff Doctor
Shigeru Yatoh, MD, PhD	Post-doc	2003-	Joslin	
Hitoshi Katsuta, MD, PhD	Post-doc	2005-	Joslin	
Esther O'Sullivan, MD	Post-doc	2005-	Joslin	
Luisa Duran	Med Stu	2006-	Joslin	
Masaki Nagaya, MD, PhD	Post-doc	2006-	Joslin	
Min-Ho Jung, MD, PhD	Post-doc	2006-	Joslin	

2. Principal Clinical and Hospital Service Responsibilities: see above

3. Major Administrative Responsibilities:

1979-1984	Director, Diabetes Clinic, Medical College of Virginia Hospital
1982-1984	Vice Chairman, Department of Internal Medicine, Medical College of Virginia School of Medicine
1984-1993	Chief, Section on Endocrinology, Diabetes, Metabolism, New England Deaconess Hospital
1984-1991	Chief, Islet Physiology Section, Elliot P. Joslin Research Laboratories
1984-1993	Medical Director, Joslin Diabetes Center
1986-	Associate Director, Diabetes and Endocrinology Research Center, Joslin Diabetes Center
1991-	Chief, Section on Islet Transplantation and Cell Biology, Elliott P. Joslin Research Laboratories
2001	Director, Diabetes Endocrinology Research Center, Joslin Diabetes Center

PART III: Bibliography

Original Reports

1. Weir GC, Martin DB. Fatty acid biosynthesis in erythrocyte. 11. Evidence in immature erythrocytes for the presence of acetyl co-enzyme carboxylase. J. Lab Clin Med. 1973; 31:37

2. Hamilton CR, Henkin RI, Weir GC, Kliman B. Olfactory status and response in clomiphene in male gonadotropin deficiency. Ann Intern Med. 1973; 78:47

3. Weir GC, Turner RD, Martin DB. Glucagon radioimmunoassay using antiserum 30K:Interference by plasma. Horm Met Res. 1973; 5:241

4. Rossini AA, Soeldner JS, Hiebert JML, Weir GC, Gleason RE. The effect of glucose anomers upon insulin and glucagon secretion. Diabetologia 1974; 10:795

5. Weir GC, Knowlton SD, Martin DB. Glucagon secretion from the perfused rat pancreas:Studies with glucose and catecholamines. J Clin Invest.1974; 54:1403

6. Weir GC, Knowlton SD, Martin DB. Somatostatin inhibition of epinephrine induced glucagon secretion. Endocrinology 1974; 95:1744

7. Weir GC, Knowlton SD, Martin DB. High molecular weight glucagon-like immuno-reactivity in plasma. J Clin Endocrinol Metab. 1975; 40:296

8. Weir GC, Knowlton SD, Martin DB, Nucleotide and nucleoside stimulation of glucagon secretion. Endocrinology 1975; 97:932

9. Weir GC, Lesser PB, Drop LJ, Fischer JE, Warshaw AL. The hypocalcemia of acute pancreatitis. Ann Intern Med. 1975; 83:185

10. Weir GC, Knowlton SD, Atkins RF, McKennan KX, Martin DB. Glucagon secretion from the perfused pancreas of streptozotocin treated rats. Diabetes 1976; 25:275-282

11. Patel YC. Weir GC. Increased somatostatin content of islets from streptozotocin diabetic rats. Clin Endocrinology 1976; 5:191-194

12. Weir GC, Goltsos PC, Steinberg EP, Patel YC. High concentration of somatostatin immunoreactivity in chicken pancreas. Diabetologia 1976;12:129 132

13. Bucher NLR, Weir GC. Insulin, glucagon, liver regeneration and DNA synthesis. Metabolism 1976; 25:1423

14. Weir GC, Atkins RF, Martin DB. Glucagon secretion from the perfused rat pancreas following acute and chronic streptozotocin. Metabolism 1976; 25:1519

15. Weir GC, Horton ES, Aoki TT, Slovik D, Jaspan J, Rubenstein AH. Secretion by glucagonomas of a possible glucagon precursor. J. Clin Invest 1977; 59:325

16. Ganda OP, Weir GC, Soeldner SJ, Gabbay K, Patel YC, Reichlin S. Ebeid AM, Legg M. A Somatostatinoma. N Eng. J. Med. 1977; 296:963-967

17. Witters LA, Ohman J, Weir GC, Lowell FC. Insulin allergy and resistance: A clinical study. Am J Med 1977; 63:703

18. Soeters PB, Weir GC, Ebeid AM, Fischer JE. Changes in insulin and glycagon with onset of hepatic encephalopathy. J. Surg Res. 1977; 23:183-188

19. Samols E, Weir GC, Ramseur R, Day JA, Patel YC. Modulation of pancreatic somatostatin by adrenergic and cholinergic agonism and by hyper and hypoglycemic sulfonamides. Metab 1977; 27:1219-1222

20. Weir GC, Samols E, Day JA Jr., Patel YC. Glucose and glucagon stimulate the secretion of somatostatin from the perfused canine pancreas. Metab 1978; 27:1223

21. Noe BD, Weir GC. Baer GE. Biosynthesis of somatostatin in pancreatic islets. Metab 1978; 27:1201

22. Noe BD, Bauer GE, Weir GC, Patel YC. Somatostatin biosynthesis in pancreatic islets: Evidence for a precursor. Endocrinology 1978; 102:1675

23. Weir GC, Samols E, Loo S, Patel YC, Gabbay KH. Somatostatin and pancreatic polypeptide secretion: Effects of glucagon, insulin and arginine. Diabetes 1979; 28:35-40.

24. Samols E, Weir GC. Adrenergic modulation of pancreatic A,B, and D cells. J. Clin Invest. 1979; 63:230-238

25. Honey RN, Weir GC. Insulin stimulates somatostatin and inhibits glucagon secretion from the perfused chicken pancreas-duodenum. Life Science 1979; 24:1747-1750

26. Bonner-Weir S, Weir GC. The organization of the endocrine pancreas: A hypo-thetical unifying view of the phylogenetic differences. General and Comparative Endocrinology 1979; 38:28-37

27. Newsome HH Jr., Weir GC, Daniel TM. Norepinephrine-secreting tumor of the organ of Zuckerland: Response to propranolol alone. JAMA 1979; 242:540-542

28. Weir GC, Schwarz JA, Mathe CJ. Inhibition of glucagon and insulin secretion from the perfused rat pancreas by a B-cell-selective somatostatin analog. Metabolism 1980; 29:68-70

29. Honey RN, Schwarz JA, Mathe CJ, Weir GC. Insulin, glucagon and somatostatin secretion from the isolated perfused rat and chicken pancreas-duodenum. Am J Physiol. 1980; 238:E150-156

30. Weir GC, Bonner-Weir S, Edwards DC. Nerve growth factor and the pancreatic APUD cells. Endoc. Res Communications 1980; 7:71-75

31. Saffouri B, Weir G, Bitar K, Makhlouf G, Stimulation of gastrin secretion from the perfused rat stomach by somatostatin antiserum. Life Sciences 1979; 25:1749-1754

32. Saffouri B, Weir GC, Bitar KN, Makhouf GM. Gastrin and somatostatin secretion by the perfused rat stomach: functional linkage of antral peptides. Am J Physiol. 1980; 238:G495-501

33. Stagner JI, Samols E, Weir GC. Sustained oscillations of insulin, glucagon, and somatostatin from the isolated canine pancreas during exposure to a constant glucose concentration. J Clin Invest 1980; 65:939-942

34. Bitar KN, Said SI, Weir GC, Saffouri B, Makhlouf GM. Neural release of vasoactive intestinal peptide from the gut. Gastroenterology 1980; 79:1288-1294

35. Honey RN, Weir GC, Acetycholine stimulates insulin, glucagon, and somatostatin release in the perfused chicken pancreas. Endocrinology 1980;107:1065-1068

36. Gazdar AF, Chick WL, Oie HK, Sims HL, King DL, Weir GC. Lauris V. Continuous clonal insulin and somatostatin secreting cell lines established from a trans-plantation rat islet cell tumor. Proc Nat Acad Sci. 1980; 77:3519-3523

37. Chick WL, Appel MC, Like AA, Weir GC, Lauris V, Porter JG, Chute RN. Serially transplantable chemically-induced rat islet cell tumor. Endocrinology 1980; 107:954-960

38. Honey RN, Fallon MB, Weir GC. Effects of exogenous insulin on somatostatin and glucagon secretion in the perfused chicken pancreas. Metabolism 1980; 29:1242-1246

39. Oyama H, Martin J, Sussman K, Weir GC, Permutt A. The biological activity of catfish pancreatic somatostatin. Regulatory Peptides 1981; 1:387-396

40. Bonner-Weir S, Trent DF, Honey RN, and Weir GC. Responses of neonatal rat islets to streptozotocin: Limited B cell regeneration and hyperglycemia. Diabetes 1981; 30:64-69

41. Bonner-Weir S, Trent DF, Zmachinski CH, Clore ET, Weir GC. Limited B cell regeneration in a B cell deficient rat model: Studies with dexamethasone. Metabolism 1981; 30:914-918

42. Weir GC, Clore ET, Zmachinski CH, Bonner-Weir S. Islet secretion in a new experimental model of non-insulin dependent diabetes mellitus. Diabetes 1981; 30:590-595

43. Trent DF, Weir GC, Heterogeneity of somatostatin-like peptides in rat brain, pancreas, and gastrointestinal tract. Endocrinology 1981; 108:2033

44. Honey RN, Weir GC, Arimura A. Stimulation of avian glucagon and insulin by somatostatin antiserum. Endocrinology 1981; 109:1971-1974

45. Samols E, Stagner JI, Weir GC, Autonomic function and control of pancreatic somatostatin. Diabetologia 1981; 20:388-392

46. DuVal JW, Saffouri B, Weir GC. Walsh JH, Arimura A, Makhlouf GM. Stimulation of gastrin and somatostatin secretion from the isolated rat stomach by bombesin. Am J Physiol 1981; 241:G242-G247

47. Lankford MV, Weir GC. Cushing's disease and failure of dexamethasone suppression. Southern Medical Journal 1981; 74:1002-1004

48. Weir GC. Non-insulin-dependent diabetes mellitus: Interplay between B cell inadequacy and insulin resistance. Am J Med. 1982; 73:461-464.(Refereed, non-invited editorial)

49. Halban PA, Bonner-Weir, S, Weir GC. Increased proinsulin biosynthesis in islets from a rat model of non-insulin dependent diabetes mellitus. Diabetes 1983; 32:277-283

50. Oie HK, Gazdar AF, Minna JD. Weir GC. Baylin SB. Clonal analysis of insulin and somatostatin secretion and L-DOPA decarboxylase expression by a rat islet cell tumor. Endocrinology 1983; 112:1070-1075

51. Fletcher DJ, Grogan WM, Weir GC. Hormone content and acute hormone release of B-cell enriched and A and D cell-enriched populations prepared by flo cytometry. Endocrinology 1982; 113:1791

52. Bonner-Weir S. Trent DF, Weir GC. Partial pancreatectomy in the rat and subsequent defect in glucose-induced insulin release. J. Clin Invest. 1983; 71: 1544-1553

53. Fletcher DJ, Trent DF, Weir GC, Catfish somatostatin is unique to piscine tissues. Regulatory Peptides 1983; 5:181-187

54. Weir GC, Halban PA, Meda P. Wollheim CB, Orci L. Renold AE. A functional and morphological study of dispersed adult pancreatic islet cells. Metabolism 1984; 33:447-453

55. Trent DF, Fletcher DJ, May JM. Bonner-Weir S, Weir GC. Abnormal islet and adipocyte function in young B-cell deficient rats with near-normoglycemia. Diabetes 1984; 33:170-174

56. Trent DF, Schwalke MA, Weir GC. Pancreatic and gut hormones during fasting; Insulin, glucagon and somatostatin. Hormone Research 1984; 19:70-76

57. Permutt MA O'Connell K, Kakita K, Malinas P, Karl I, Bonner-Weir S., Weir GC. Giddings SJ. An in vitro analyses of pancreatic protein and insulin biosynthesis in a rat model for non-insulin dependent diabetes. J Clin Invest 1984; 73:1344-1350

58. Trimble ER, Weir GC, Gjinovci A. Assimacopoulos-Jeannet F, Benzi R, Renold AE. Increased insulin responsiveness in vivo and in vitro consequent to induced hyperinsulinemia in the rat. Diabetes 1984; 33:444-449

59. Leahy JL, Bonner-Weir S, Weir GC. Abnormal glucose regulation of insulin secretion in models of reduced B cell mass. Diabetes 1984; 33:667-673

60. Giddings SJ, Orland MG, Weir GC, Bonner-Weir S., Permutt MA. Impaired insulin biosynthetic capacity in a rat model for non-insulin dependent diabetes.Studies with dexamethasone. Diabetes 1985; 34:235-240

61. Leahy JL, Weir GC. Unresponsiveness to glucose in a streptozocin model of diabetes: Inappropriate insulin and glucagon responses to a reduction of glucose concentration. Diabetes 1985; 34:653-659

62. Leahy JL, Bonner-Weir S, Weir GC. Abnormal insulin secretion in a streptozocin model of diabetes: Effects of insulin treatment, Diabetes 1985; 34:660-666

63. Colella RM, Bonner-Weir S, Braunstein LP, Schwalke M, Weir GC. Pancreatic Islets of variable size-insulin secretion and glucose utilization. Life Sciences 1985; 37:1059-1065

64. Leahy JL, Cooper HE, Deal DA, and Weir GC. Chronic hyperglycemia is associated with impaired glucose influences on insulin secretion. A study in normal rats using chronic in vivo glucose infusions. J. Clin Invest. 1986; 77:908-915

65. Weir GC, Leahy JL, Bonner-Weir S. Experimental reaction of B-cell mass: Implications for the pathogenesis of diabetes. Diabetes/Metabolism Reviews 1986; 2:125-161

66. Weir GC, Leahy JL, Barras E. Braunstein LP. Characteristics of insulin and glucagon release from the perfused pancreas, intact isolated islets, and dispersed cells. Hormone Res. 1986; 24:62-72

67. Wagman LD, Newsome HH, Miller KB, Thomas RB, Weir GC. Effect of acute discontinuation of total parentaeral nutrition. Ann Surger. 1986; 204: 524-529

68. Leahy JL, Cooper, HE, Weir GC. Impaired insulin secretion associated with nearnormoglycemia: A study in normal rats using 96 hour in vivo glucose infusions. Diabetes 1987; 36:459-64

69. Colella RM, May JM, Bonner-Weir S, Leahy JH, Weir GC. Glucose utilization in islets of hyperglycemic rat models with impaired glucose-induced insulin secretion. Metabolism 1987; 36:335-337

70. Mojov S, Weir GC, Habener JF. Insulinotropin: Glucagon-like peptide I(7-37) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas. J Clin Invest. 1987; 79:616-619

71. Brockenbrough JS, Weir GC, Korc M. Alterations in EGF binding to acini during pancreatic regeneration in the rat. Int. J. Pancreatology 1988; (3:415-424)

72. Leahy JL, Weir GC. Evolution of abnormal insulin secretory responses during 48 hours of in vivo hyperglycemia. Diabetes 1988; 37:217-222

73. Brockenbrough JS, Weir, GC, Bonner-Weir S. Discordance of exocrine and endocrine growth following 90% partial pancreatectomy in the rat. Diabetes 1988; 37:232-236

74. Leahy JL, Bonner-Weir S, Weir GC, Minimal chronic hyperglycemia is a critical determinant of impaired insulin secretion following incomplete pancreatectomy in the rat. J. Clin. Invest. 1988; 77:1407-1414

75. Bonner-Weir S, Deery D, Leahy JL, Weir GC. Compensatory growth of pancreatic B-cells in adult rats after short-term (96 hours) glucose infusion. Diabetes 1989; 38:49-53

76. Weir GC, Mojsov S, Hendrick GK, Habener JF. Glucagon like Peptide-1(7-37) actions on endocrine pancreas. Diabetes 1989; 38:338-342

77. Gross G, Skvorak A, Hendrick G, Weir G, Villa-Komaroff L, Halban P. Oxidation of rat insulin II, but not I, leads to anomalious elution profiles upon HPLC analysis of insulin related peptides. FEBS Letters 1989; 241:205-208

78. Lee HC, Bonner-Weir S, Weir GC, Leahy JL. Compensatory adaption to partial pancreatectomy and glucocorticoid challenge in the rat. Endocrinology 1989; 124:1571-1575

79. Gross D.J., Halban PA, Kahn CR, Weir GC, Villa-Komaroff L. Partial diversion a mutant proinsulin (B10 aspartic acid) from the regulated to the constitutive secretory pathway in transfected AtT-20 cells. Proc. Natl. Acad.Sci USA 1989; 86:4107-4111

80 Gross DJ, Villa-Komaroff L, Kahn CR, Weir GC., Halban PA Deletion of a highly conserved tetrapeptide sequence of the proinsulin connecting peptide (C-peptide) inhibits proinsulin to insulin conversion by transfected pituitary corticotroph (AtT20) cells. J. Biol. Chem. 1989; 264:21486-21490

81. Gefel D, Hendrick GK, Mojsov S, Habener J, Weir GC. Glucagon like peptide-I analogs; effects on insulin secretion and adenosine 3',5'-monophosphate formation. Endocrinology 1990; 126:2164-2168

82. Thorens B, Weir GC, Leahy JL, Lodish HF, Bonner-Weir S. Reduced expression of the liver/beta cell glucose transporter isoform in glucose-insensitive pancreatic beta cells of diabetic rats. Proc. Natl. Acad Sci. USA 1990; 87:6492-6496

83. Leahy JL, Halban PA, Weir GC. Relative hypersection of proinsulin in rat model of NIDDM. Diabetes 1991; 40:985-989

84. Smith FE, Rosen KM, Villa-Komaroff L, Weir GC. Bonner-Weir S. Enhanced insulin-like growth factor I gene expression in regenerating rat pancreas. Proc Natl. Acad Sci USA 1991; 88:6152-6156

85. Leahy JL, Weir GC. B-cell dysfunction in hyperglycemic rat models: recovery of glucose-induced insulin secretion with lowering of the ambient glucose level. Diabetologia 1991; 34:620-647

86. Thorens B, Wu Y-J, Leahy JL, Weir GC. The loss of GLUT-2 expression by glucose-unresponsive beta cells of db/db mice is reversible and is induced by the diabetic environment. J. Clin. Invest. 1992; 90:77-85

87. Tal M, Wu Y-J, Leiser M, Surana M, Lodish H, Fleischer N, Weir G, Efrat S. (Val 12) HRAS downregulates GLUT-2 in beta cells of transgenic mice without affecting glucose homeostasis. Proc. Natl. Acad Sci. USA 1992; 89:5744-5748

88. Hendrick GK, Gjinovci A, Baxter LA, Mojsov S, Wollheim CB, Habener JF, Weir GC. Glucagon-like peptide I-(7-37) suppresses hyperglycemia in rats. Metabolism 1992; 42:1-6

89. Chen C, Thorens B, Bonner-Weir S, Weir GC, Leahy JL. Recovery of glucose-induced insulin secretion in a rat model of non-insulin dependent diabetes is not accompanied by return of the B-cell GLUT-2 glucose transporter. Diabetes 1992; 41:1320-1327

90. Schuppin GT, Bonner-Weir S, Montana E, Kaiser N, Weir G.C. Replication of adult pancreatic B-cells cultured on bovine corneal endothelial extracellular matrix. In Vitro 1993; 29A:339-344

91. Montana E, Bonner-Weir S, Weir GC. Beta cell mass and growth after syngeneic islet cell transplantation in normal and streptozocin diabetic C57 BL/6 mice. J. Clin. Invest. 1993; 91:780-787

92. Montana E, Bonner-Weir S, Weir GC. Transplanted beta cell response to increased metabolic demand: Changes in beta cell replication and mass. J Clin Invest. 1994; 93:1577-1582

93. Efrat S, Leiser M, Wu Y-J, Fusco-DeMane D, Emran OA, Sunra M, Jetton JL, Magnuson MA, Weir GC, Fleischer N. Ribozyme-mediated attentuation of pancreatic B-cell glucokinase expression in transgenic mice results in impaired glucose-induced insulin secretion. Proc Nat Acad Sci. 1994; 91:2051-2055

94. Lorenzo A, Razzaboni B, Weir GC, Yankner BA. Pancreatic islet toxicity of amylin associated with type-2 diabetes mellitus. Nature. 1994; 368:756-760

95. Juang, J-H, Bonner-Weir S, Wu Y-J, Weir GC. Beneficial influence of normoglycemic environment upon the growth and function of transplanted islets. Diabetes 1994; 43:1334-1339

96. Davalli AM, Ogawa Y, Scaglia L, Wu Y-J, Hollister J, Bonner-Weir S, Weir GC. Function, mass, and replication of porcine and rat islets transplanted into diabetic nude mice. Diabetes 1995; 44:104-111

97. Davalli AM, Ogawa Y, Ricordi C, Scharp DW, Bonner-Weir S, Weir GC. Selective decrease in the beta cell mass of human islets transplanted into diabetic nude mice. Transplantation 1995; 59:817-820

98. Ogawa Y, Noma Y, Davalli AM, Wu Y-J, Thorens B, Bonner-Weir S, Weir GC. Loss of glucose-induced insulin secretion and GLUT2 expression in transplanted beta cells. Diabetes 1995; 44:75-79

99. Noma Y, Bonner-Weir S, Latimer JB, Davalli AM, Weir GC. Translocation of glucokinase in pancreatic beta cells during acute and chronic hyperglycemia. Endocrinology 1996; 137:1485-1491

100. Juang J-H, Bonner-Weir S, Ogawa Y, Vacanti JP, Weir GC. Outcome of subcutaneous islet transplantation improved by polymer device. Transplantation 1996; 61:1557-1561

101. Davalli AM, Napoli R, Weitgasser R, Capotorto JV, Hirshman MF, Finegood DT, Bonner-Weir S, Horton ES, Weir GC. Long-term normalization of GLUT-4 protein content in skeletal muscle of diabetic rats following islet transplantation. J Endocrinology 1996; 150(2):255-263

102. Davalli AM, Scaglia L, Zangen DH, Hollister J, Bonner-Weir S, Weir GC. Islet vulnerability in the immediate post-transplant period. Role exerted by the recipients metabolic control. Diabetes 1996; 45:1161-1167

103. Napoli R, Davalli AM, Hirshman MF, Weitgasser R, Weir GC, Horton ES. Islet transplantation under the kidney capsule fully corrects impaired skeletal muscle glucose transport system in streptozocin diabetic rats. J Clin Invest. 1996; 97:1389-1397

104. Verchere CB, D'Alessio DA, Palmiter RD, Weir GC, Bonner-Weir S, Baskin DG, Kahn SE. Islet amyloid formation associated with hyperglycemia in transgenic mice with pancreatic beta cell expression of human islet amyloid polypeptide. Proc Natl Acad Sci USA 1996; 93:3492-3496

105. Lipes MA, Cooper E, Skelly R, Rhodes CJ, Boschetti E, Weir GC, Davalli AM. Transgenic engineering of non-islet cells for beta cell replacement in insulin dependent diabetes mellitus. Proc Natl Acad Sci USA 1996; 93:8595-8600

106. Suzuki K, Bonner-Weir S, Hollister J, Weir GC. A method of estimating number and mass of islets transplanted within a membrane device. Cell Transplantation 1996; 5:613-625

107. Kieffer TJ, Heller RS, Weir GC, Habener JF. Distribution of glucagon receptors on hormone-specific endocrine cells of rat pancreatic islets. Endocrinology 1996; 137:5119-5125

108. Weitgasser R, Davalli AM, Capotorto JV, Finegood DT, Bonner-Weir S, Weir GC. Islet transplantation in diabetic Lewis rats -A comparison of the transplantation sites kidney and spleen capsule. Acta Med. Austriaca 1996; 23:156-159

109. Zangen DH, Bonner-Weir S, Lee CH, Latimer JB, Miller CP, Habener JF, Weir GC. Reduced insulin, GLUT2 and IDX-1 in beta cells after partial pancreatectomy. Diabetes 1997; 46:258-264

110. Weir GC, Bonner-Weir S. Scientific and political impediments to successful islet transplantation. Diabetes 1997; 46:1247-1256

111. Weir GC, Sharma A, Zangen DH, Bonner-Weir S. Transcription factor abnormalities as a cause of beta cell dysfunction in diabetes: A hypothesis. Acta Diabetologica 1997; 34:177-184

112. Suzuki K, Bonner-Weir S, Hollister-Lock J, Colton CK, Weir GC. Number and volume of islets transplanted in immunobarrier devices. Cell Transplantation 1998; 7:47-52

113. Seufert JR, Weir GC, Habener JF. Differential expression of the insulin gene transcriptional repressor C/EBPbeta and transactivatior IDX-1 in rat pancreatic beta cells during the development of diabetes mellitus. J Clin Invest. 1998; 101:2528-2539

114. Suzuki K, Bonner-Weir S, Trivedi N, Yoon K-H, Hollister-Lock J, Colton CK, Weir GC. Function and survival of macroencapsulated syngeneic islets transplanted into streptozocin-diabetic mice. Transplantation 1998; 66:21-28

115. Vasir B, Aiello LP, Yoon K-H, Quickel RR, Bonner-Weir S, Weir GC. Hypoxia induced VEGF gene and protein expression in rat islets. Diabetes 1998; 47:1894-1903

116. Sharma A, Zangen DH, Reitz P, Taneja M, Lissauer ME, Smith FE, Miller CP, Weir GC, Habener JF, Bonner-Weir S. The homeodomain protein IDX-1 increases after an early burst of proliferation during pancreatic regeneration. Diabetes 1999; 48:507-513

117. Tatarkiewicz K, Hollister-Lock J, Quickel RR, Colton CK, Bonner-Weir S, Weir GC. Reversal of hyperglycemia in mice after subcutaneous transplantation of macroencapsulated islets. Transplantation 1999; 67:665-671

118. Finegood DT, Weir GC, Bonner-Weir S. Prior streptozotocin treatment does not inhibit pancreas regeneration. Am J Physiol 1999; 276:E822-E827

119. Giorgino F, Logoluso F, Davalli A, Napoli R, Laviola L, Hirshman MF, Horton ES, Weir GC, Smith RJ. Islet transplantation restores normal levels of insulin receptor and substrate tyrosine phosphorylation and phosphatidylinositol 3-kinase activity in skeletal muscle and myocardium of streptozocin diabetic rats. Diabetes 1999; 48:801-812

120. Lee B, Jonas JC, Weir GC, Laychock SG. Glucose regulates expression of inositol 1,4,5-triphosphate receptor isoforms in isolated rat pancreatic islets. Endocrinology 1999; 140:2173-2182

121. Yoon K-H, Quickel RR, Tatarkiewicz K, Ulrich TR, Hollister-Lock J, Trivedi N, Bonner-Weir S, Weir GC. Differentiation and expansion of beta cell mass in porcine neonatal pancreatic cell clusters transplanted into nude mice. Cell Transplantation 1999; 8:673-689

122. Jonas J-C, Sharma A, Hasenkamp W, Ilkova H, Patane G, Laybutt R, Bonner-Weir S, Weir GC. Chronic hyperglycemia triggers loss of pancreatic beta cell differentiation in an animal model of diabetes. J. Biol. Chem. 1999; 274:14112-14121

123. Feng S, Quickel RR, Hollister-Lock J, McCloud M, Bonner-Weir S, Mulligan R, Weir GC. Prolonged xenograft survival of islets infected with small doses of adenovirus containing CTLA4Ig. Transplantation 1999; 67:607-1613

124. Wu H, Avgoustiniatos ES, Swette L, Bonner-Weir S, Weir GC, Colton CK. In situ electrochemical oxygen generation with an immunoisolation device. New York Acad Science 1999; 875:105-125

125. Weitgasser R, Davalli AM, Weir GC. Measurement of glucose concentrations in rats: Differences between glucose meter and plasma laboratory results. Diabetologia 1999; 42:256

126. Trivedi N, Steil GM, Colton CK, Bonner-Weir S, Weir GC. Improved vascularization of planar diffusion membrane devices following continuous infusion of vascular endothelial growth factor. Cell Transplantation 2000; 9:115-124

127. de Souza CJ, Capotorto JV, Cornell-Kennon S, Wu Y-J, Steil GM, Trivedi N, Weir GC. The beta-cell dysfunction in 48-hour glucose infused rats is not a consequence of elevated plasma lipid or islet triglyceride levels. Metabolism 2000; 49:755-759

128. Vasir B, Reitz P, Xu Gang, Sharma A, Bonner-Weir S, Weir GC. Diabetic state delays gene expression of markers of angiogenesis HGF, uPA and uPAR in transplanted islets. Diabetologia 2000; 43:763-772

129. Bonner-Weir S, Taneja M, Weir GC, Tatarkiewicz, Song K-H, Sharma A, O'Neil J. In vitro cultivation of human islets from expanded ductal tissue. PNAS 2000; 97:7999-8004

130. Steil GM, Trivedi N, Jonas J-C, Hasenkamp WM, Sharma A, Bonner-Weir S, Weir GC. Enhanced beta-cell function with normal islet gene expression following adaptation of β-cell mass to substrate oversupply. Am J Physiol Endoc Metab. 2001; 280:E788-E796

131. Trivedi N, Keegan M, Steil GM, Hollister-Lock J, Hasenkamp W, Colton CK, Bonner-Weir S. Weir GC. Islets in alginate macrobeads reverse diabetes despite minimal acute insulin secretory responses. Transplantation 2001; 71:203-211

132. Weir GC, Laybutt DR, Kaneto H, Bonner-Weir S, Sharma S. ß-Cell Adaptation and Decompensation During the Progression of Diabetes. Diabetes 2001; 50, suppl 1:S154S159

133. Vasir B, Jonas J-C, Steil GM, Hollister-Lock J, Hasenkamp W, Sharma A, Bonner-Weir S, Weir GC. Gene expression of VEGF and its receptors Flk-1/KDR and Flt-1 in cultured and transplanted rat islets. Transplantation 2001; 71:924-935

134. Trivedi N, Hollister-Lock J, Lopez-Avalos MD, O'Neil JJ, Keegan M, Bonner- Weir S, Weir GC. Increase in β-cell mass after transplantation of porcine neonatal pancreatic cell clusters is due to proliferation of β- cells and differentiation of duct cells. Endocrinology 2001; 142:2115-2122

135. Jonas JC, Laybutt DR, Steil GM, Trivedi N, Pertusa JG, Van de Casteele M, Weir GC and Henquin JC. High glucose stimulates early response gene c-Myc expression in rat pancreatic β-cells. J Biol Chem. 2001; 276:35375-35381

136. Lopez-Avalos MD, Tatarkiewicz K, Sharma A, Bonner-Weir S and Weir GC. Enhanced maturation of porcine neonatal pancreatic cell clusters with growth factors fails to improve transplantation outcome. Transplantation 2001; 71:1154-1162

137. Tatarkiewicz K, Garcia M, Lopez-Avalos M, Bonner-Weir S and Weir GC. Porcine neonatal pancreatic cell clusters in tissue culture: Benefits of serum and immobilization in alginate hydrogel. Transplantation 2001; 71:1518-1526

138. Tatarkiewicz K, Garcia M, Omer A, van Schilfgaarde R, Weir GC, and de Vos P. C-peptide responses after meal challenge in mice transplanted with microencapsulated rat islets. Diabetologia 2001; 44:646-653

139. Laviola L, Belsanti G, Davalli AM, Napoli R, Perrini S, Weir GC, Giorgino R, Giorgino F. Effects of streptozocin diabetes treatment by islet transplantation on in vivo insulin signaling in rat heart. Diabetes 2001; 50:2709-2720

140. Papas KK, Colton CK, Gounarides JS, Roos ES, Jarema MAC, Shapiro MJ, Cheng LL, Cline GW, Shulman GI, Wu H, Bonner-Weir S, Weir GC. NMR spectroscopy in beta cell engineering and islet transplntation. Bioartificial Organs III: Tissue Sourcing, Immunoisolation and Clinical Trials. Ann NY Acad of Sciences 2001; 944:96-119

141. Kaneto H, Xu G, Song K-H, Suzuma K, Sharma S, Bonner-Weir S, Weir GC. Activation of the Hexosamine Pathway Leads to Deterioration of Pancreatic Beta-Cell Function through the Induction of Oxidative Stress. J Biol Chem. 2001; 276:31099-31104

142. Duvivier-Kali VF, Omer A,. Parent RJ, O'Neil JJ, Weir GC. Complete protection of islets against allorejection and autoimmunity by a simple barium-alginate membrane. Diabetes 2001; 50:1698-1705

143. Bühler L, Deng S, O'Neil J, Kitamura H Koulmanda M, Alwayn I, Appel J, Awwad M, Sachs DH, Weir G, Squifflet JP, Cooper DKC, Morel P. Adult porcine islet transplantation in baboons treated with conventional immunosuppression or a nonmyeloablative regimen and CD154 blockade. Xenotransplantation 2002; 9:3-13

144. Laybutt DR, Weir GC, Kaneto H, Palmiter RD, Sharma S, Bonner-Weir S. Overexpression of c-Myc in Beta-Cells of Transgenic Mice Causes Proliferation and Apoptosis, Downregulation of Insulin Gene Expression and Diabetes 2002; 51:1793-1804

145. Kaneto H, Suzuma K, Sharma A, Bonner-Weir S, King, GL and Weir GC. Involvement of Protein Kinase C β2 in *c-myc* Induction by High Glucose in Pancreatic β-Cells. J Biol Chem. 2002; 277:3680-3685

146. Laybutt DR, Kaneto H, Hasenkamp W, Grey S, Jonas J-C, Sgroi DC, Groff A, Ferran C, Sharma A, Bonner-Weir S and Weir GC. Increased Expression of Antioxidant and Antiapoptotic Genes in Islets that may Contribute to Beta-Cell Survival During Chronic Hyperglycemia. Diabetes 2002; 51:413-423

147. Laybutt DR, Sharma A, Sgroi DC, Gaudet J, Bonner-Weir S, Weir GC. Genetic Regulation of Metabolic Pathways in β-cells Disrupted by Hyperglycemia. J Biol Chem. 2002; 277:10912-10921

148. Kaneto H, Sharma A, Suzuma K, Laybutt DR, Xu G, Bonner-Weir S, Weir GC. Induction of c-Myc Expression Suppresses the Insulin Gene Transcription by Inhibiting NeuroD/BETA2-mediated Transcriptional Activation. J Biol Chem. 2002; 277:12998-13006

149. Matarazzo M, Giardina MG, Guardasole, Davalli AM, Horton ES, Weir GC, Sacca L, Napoli R. Islet transplantation under the kidney capsule corrects the defects in glycogen metabolism in both liver and muscle of streptozocin diabetic rats. Cell Transplantation 2002; 11:103-112

150. Makhlouf L, Duvivier-Kali V, Bonner-Weir S, Weir, GC, Sayegh MH. Importance of glycemic control on the primary function of islet transplants. Transplantation 2003; 76: 657-664

151. Kaneto H, Xu G, Fujii N, Kim S, Bonner-Weir S, Weir GC. Involvement of c-Jun N-terminal Kinase in Oxidative Stress-mediated Suppression of Insulin Gene Expression. J Biol Chem. 2002; 277:30010-30018

152. Omer A, Keegan M, Czismadia E, De Vos P, Van Rooijen N, Bonner-Weir S, Weir GC. Macrophage depletion improves survival of porcine neonatal pancreatic cell clusters contained in alginate macrocapsules transplanted into rats. Xenotransplantation 2003; 10:240-251

153. Omer A, F. Duvivier-Kali VF, Trivedi N, Wilmot K, Bonner-Weir S, Weir GC. Survival and maturation of microencapsulated porcine neonatal pancreatic cell clusters transplanted into immunocompetent diabetic mice. Diabetes 2003; 52:69-75

154. Tatarkiewicz K, Lopez-Avalos MD, Yoon KH, Trivedi N, Quickel RR, Bonner-Weir S, Weir GC. Development and retroviral transduction of porcine neonatal pancreatic islet cells in monolayer culture. Development Growth and Differentiation 2003; 45:39-50

155. Laybutt DR, Glandt M, Xu G, Ahn YB, Trivedi N, Bonner-Weir S, Weir GC. Critical Reduction in Beta-Cell Mass Results in Two Distinct Outcomes Over Time: Adaptation With Impaired Glucose Tolerance or Decompensated Diabetes. J Biol Chem. 2003; 278:2997-3005

156. Patane G, Kaneto H, Toschi E, Sharma A, Gupta S, Weir G. Induction of Mad expression leads to augmentation of insulin gene expression. Biochem Biophys Res Com. 2003; 303:1199-1208

157. Noguchi H, Kaneto H, Weir GC, Bonner-Weir S. PDX-1 protein containing its own Antennapedia-like protein transduction domain can transduce pancreatic duct and islet cells.Diabetes 2003; 52:1732-1737

158. Yoon JC, Xu G, Deeney JT, Yang S-N, Rhee J, Puigserver P, Levens AR, Yang R, Zhang C-Y, Lowell BB, Berggren P-O, Newgard CB, Bonner-Weir S, Weir GC, Spiegelman BM Suppression of beta cell energy metabolism and insulin release by PGC-1alpha. Developmental Cell 2003; 5:73-83

159. Omer A, Duvivier-Kali VF, Aschenbach W, Tchipashvili V, Laurie J. Goodyear LJ, Weir GC. Exercise induces hypoglycemia in rats with islet transplants. Diabetes 2004; 53:360-365

160. Ko SH, Suh SH, Kim BJ, Ahn YB, Song KH, Yoo SJ, Son HS, Cha BY, Lee KW, Son HY, Kang SK, Bonner-Weir S, Weir GC, Yoon KH, Park CG. Expression of the intermediate filament vimentin in proliferating duct cells as a marker of pancreatic precursor cells. Pancreas 2004; 28:121-128

161. Fernandes J, Duvivier-Kali VF, Keegan M, Hollister-Lock J, Omer A, Su S, Bonner-Weir S, Feng S, Lee J-S, Mulligan RC, Weir GC. Transplantation of Islets Transduced with CTLA4-Ig and TGFβ Using Adenovirus and Lentivirus Vectors. Transplant Immunology 2004; 13:191-200

162. Weir, GC. Can we make surrogate β-cells better than the original? Seminars in Cell & Developmental Biology 2004; 15:347-357

163. Duvivier-Kali VF, Omer A, Lopez-Avalos MD, O'Neil JJ, Weir GC. Survival of microencapsulated adult pig islets in mice in spite of an antibody response. Am J Transplantation 2004; 4:1991-2000

164. Weir GC, Bonner-Weir S. Five stages of evolving beta cell dysfunction during progression to diabetes. Diabetes 2004; 53, Suppl 3:S16-21

165. Weir GC, Bonner-Weir S. Beta-cell precursors--a work in progress. Nature Biotechnology 2004; 22:1095-6

166. Omer A, Duvivier-Kali VF, Fernandez J, Tchipashvili V, Colton CK, Weir GC. Long term normoglycemia in rats transplanted with encapsulated islets. Transplantation 2005; 79:52-58

167. Akari I, Weir GC, Bonner-Weir S. Induced ICER Igamma down-regulates cyclin A expression and cell proliferation in insulin-producing beta cells. Biochem Biophys Res Comm. 2005; 329:925-9

168. Iype T, Garmey FJ, Schisler JC, Nesher R, Weir GC, Becker TC, Newgard CB,,Griffen SC, Mirmira RG. Mechanism of Insulin Gene Regulation by the Pancreatic Transcription Factor Pdx-1: Application of pre-mrna analysis and chromatin immunoprecipitation to assess formation of functional transcriptional complexes. J Biol Chem. 2005; 280:16798-807.

169. Schisler JC, Jensen PB, Taylor DG, Becker TC, Krag FK, German M, Weir GC, Lu D, Raghavendra GM, and Newgard CB. The Nkx6.1 homeodomain transcription factor directly suppresses glucagon expression and regulates glucose-stimulated insulin secretion in islet beta–cells. Proc Nat Acad Sci. 2005; 102:7297-7302

170. King A, Lock J, Xu G, Bonner-Weir S, Weir GC. Islet transplantation outcomes are better with fresh islets and exendin-4 treatment. Diabetologia 2005; 48:2074-9

171. Inada A, Nagai K, Arai H, Miyazaki JI, Nomura K, Kanamori H, Yamada Y, Bonner-Weir S, Weir GC, Fukatsu A, Seino Y. Establishment of a diabetic mouse model with progressive diabetic nephropathy. Am J Path. 2005; 167:327-336

172. Bonner-Weir S, Weir GC. New sources of pancreatic beta cells. Nature Biotechnology 2005; 23:857-861

173. Gaglia JL, Shapiro AMJ, Weir GC. Islet transplantation: Progress and challenge. Archives of Medical Research 2005; 36:273-280

174. Fukui K, Yang Q, Cao Y, Takahashi N, Hatakeyama H, Wang H, Wada J, Zhang Y, Marselli L, Nammo T, Yoneda K, Onishi M, Higashiyama S, Matsuzawa Y, Gonzalez FJ, Weir GC, Kasai H, Shimomura I, Miyagawa J, Wollheim CB, Yamagata K. The HNF-1 target collectrin controls insulin exocytosis by SNARE complex formation. Cell Metab. 2005; 2:373-84

175. Park S, Dong X, Fisher TL, Dunn SL, Omer AK, Weir G, White MF. Exendin-4 promotes IRS2 signaling to mediate pancreatic beta-cell growth and function. J Biol Chem. 2005; 281:1159-1168

176. Papas KK, Avgoustiniatos ES, Tempelman LA, Weir GC, Colton CK, Pisania A, Rappel MJ, Friberg AS, Bauer AC, Hering BJ. High-density culture of human islets on top of silicone rubber membranes. Transplant Proc. 2005; 37:3412-3414

177. Koulmanda M, Smith N, Qipo A, Weir G, Auchincloss H, and Strom TB. Prolonged survival of allogeneic islets in cynomolgus monkeys after short-term anti-CD154-based therapy: nonimmunologic graft failure? Am J Transplant. 2006; 6:687-96

178. López-Avalos MD, Duvivier-Kali VF, Xu G, Sharma A, Weir GC. The ubiquitin-proteasome pathway in pancreatic islets. Diabetes 2006; 55:1223-1231

179. Xu G, Kaneto H, Lopez-Avalos MD, Weir GC, Bonner-Weir S. GLP-1/exendin-4 facilitates beta-cell neogenesis in rat and human pancreatic ducts. Diabetes Res Clin Pract 2006; 73:107-110

180. Weir GC, Bonner-Weir. A dominant role for glucose in β-cell compensation of insulin resistance. J Clin Invest. 2007; 117:81-83

181. Ahn YB, Xu G, Marselli L, Toschi E, Sharma A, Bonner-Weir S, Sgroi DC, Weir GC. Changes in gene expression in beta cells after islet isolation and transplantation using laser capture microdissection. Diabetologia 2007;.50:334-342

182. Yatoh S, Akashi T, Chan PP, Kaneto H, Sharma A, Bonner-Weir S, Weir GC. NeuroD and reaggregation induce β-cell specific gene expression in cultured hepatocytes. Diabetes Metab Res Rev 2007; 23:239-249

183. Yatoh S, Dodge R, Akashi T, Omer A, Sharma A, Weir GX, Bonner-Weir S. Differentiation of purified human pancreatic duct cells to β–cells. 2007; Submitted

184. Cagliero E, Martha Pavlakis M, Anil Chandraker A, Arthur Dea A, Heather Turgeon H, Omer A, David M. Nathan DM, Auchincloss H, Weir GC. Islet After Kidney Transplantation Using the Edmonton Protocol: a Single Center Experience. 2007; Submitted

185. Xu G, Kaneto H, Laybutt DR, Duvivier-Kali VF, Suzuma K, King GL, Weir GC, Bonner-Weir S. Downregulation of GLP-1 and GIP Receptor Expression by Hyperglycemia: Possible Contribution to Impaired Incretin Effects in Diabetes. 2007; In press.

186. Klearchos K. Papas KK, , Colton CK, Nelson RA, Rozak PR, Avgoustiniatos ES, Scott WE, Wildey GM, Pisania A, Weir GC, Hering BJ. Human Islet Oxygen Consumption Rate and DNA Measurements Predict Diabetes Reversal in Nude Mice. Am J Transplant 2007

187. Papas KK, Pisania A, Wu H, Weir GC, Colton CK ,
A Stirred Microchamber for Oxygen Consumption Rate Measurements with Pancreatic Islets. Biotechnology and Bioengineering 2007, in press

188. Arikawa E, Ma RC, Isshiki K, Luptak I, He Z, Yasuda Y, Maeno Y, Patti ME, Weir GC, Harris RA, Zammit VA, Tian R, King GL. Effects of Insulin Replacements, Inhibitors of

Angiotensin, and PKCb's Actions to Normalize Cardiac Gene Expression and Fuel Metabolism in Diabetic Rats. Diabetes. 2007, 56:1410-1420.

189. Pisania A, Papas KK, Rappel MJ, Powers DE, Weir GC, Colton CK. A Quantitative Membrane Integrity Test for Islets of Langerhans. 2007; Submitted

190. Anna Pisania, Klearchos K. Papas, Daryl E. Powers, Michael J. Rappel, Abdulkadir Omer, Susan Bonner-Weir, Gordon C. Weir, Clark K. ColtonEnumeration of islets by nuclei counting and light microscopic analysis. 2007; Submitted

191. O'Neil JJ, Omer A, Tchipashvili V, Lei J, Pisania A, Colton CK, Weir GC, Bonner-Weir S. Quantitative analysis of cell composition of human pancreatic islet preparations. 2007; Submitted

192. Pisania A, Bonner-Weir S, Weir G, Colton CK. Comparison of light and electron microscopy for measuring purity of human pancreatic islet preparations. 2007; Submitted

Reviews and Letters

1. Muller WA, Weir GC, Pathophysiology of glucagon secretion (Review) In:Ioachim KL ed. Pathobiology Annual. New York: Appleton-Century-Crofts 1974; 263

2. Muller WA, Weir GC. Pathophysiology of glucagon secretion. In: Sommers SC ed. Endocrine Pathology Decennial 1966-1975. New York: Appleton-Century Crofts 1975; 363-390

3. Novelline, RA, Krudy, AG, Waltman, Weir GC. Athanasoulis CA. The glucagons syndrome: A dermatologic presentation-An angiographic diagnosis. Exhibit: Annual Convention of the American Medical Association, Dallas, Texas 1976 JAMA 1976; 235-1796

4. Weir GC, Novelline RA. The glucagonoma syndrome. American Family Physician 1977; 16:83-84

5. Weir GC, Pancreas: Insulin, glucagon, gastrin somatostatin. In: AltmanPL, Katz DD. Federation of American Societies for Experimental Biology Bethesda 1977; 323-326

6. Weir GC, Bonner-Weir S. Somatostatin: Its diverse physiological roles and diverse therapeutic implications. Hospital Formulary 1977. 1976; 12:157-161

7. Weir GC, Heterogeneity of glucagon in plasma. In: Foa PP, Bajaj JS, Foa NL eds. Glucagon-Its role in physiology and clinical medicine. New York Springer Verlag 1977; 65-76

8. Weir GC, Bonner-Weir S. Somatostatin: diverse physiological role and diverse therapeutic implications. Medical College of Virginia Quarterly 1977; 13:5-11

9. Weir GC. Glucagon in normal physiology and diabetes mellitus. In: Brownlee M ed. Diabetes Mellitus vol. 1. New York: Garland STPM Press 1981; 207-260

10. Samols E, Weir G, Bonner-Weir S. "Intraislet insulin-glucagon-somatostatin relationships". In: Lefebvre P ed. Handbook of Experimental Pharmacology GG/II Berlin: Springer-Verlag 1983; 133-173

11. Weir GC. "Cyclic nucleotides in the control of glucagon secretin". In:Lefevre P ed. Handbook of Experimental Pharmacology GG/II. Berlin:Springer-Verlag 1983; 75-82

12. Fletcher DJ, Weir GC. Tissue culture of dispersed islet cells. In: Larner J. Pohl S eds. Methods in Diabetes Research. New York: John Wiley & Sons, Inc, 1984;

13. Weir GC and Bonner-Weir S. In Patel YC, Tannenbaum GS, eds. Pancreatic Somatostatin Adv. Exp. Med. 1985; 188:403-423

14. Samols E, Bonner-Weir S, Weir GC. Insulin-glucagon-somatostatin relationships. Clinic in Endocrinology and Metabolism 1986; 15:33-58

15. Halford BN, Weir GC. Ingested carbohydrates and blood glucose in diabetics. JAMA 1985; 254:234

16. Marble A, Selenkow HA, Rose LI, Dluhy RG, Weir GC, Williams GH. Endocrine Diseases In: Avery GS, ed., Drug Treatment, Adis press Ltd, 1986 (in press). Speight TM, ed. Avery's Drug Treatment 3rd Edition. Adis Press Ltd, 1987; 522-590

17. Weir GC, O'Hare JA. Evaluation of control metabolic control in diabetes. In: Becker KL, ed. Principles and Practice of Endocrinology and Metabolism. Lippencott 1990; 1094-1096

18. O'Hare JA, Weir GC. Insulin therapy. 1990; 1102-1109

19. Ganda OP, Weir GC. Oral hypoglycemia agents. Ibid 1990; 1099-1102

20. Halban PA, Weir GC. Islet cell hormones: Production and degradation. Ibid 1990; 1068-1073

21. Weir, GC, Eisenbarth GS. Etiology of Diabetes Mellitus. In Zhi-Sheng Chi, ed. Diabetiology (Beijing, China) 1987 (in press)

22. Habener JF, Mojsov S, Drucker DJ, Philippe J. Weir GC. Cell specific alternative processing of proglucagon. In: Thorn NA, Treiman M. Peterson OH. Alfred Benzon Symposium 25. Copenhagen: Munksgaard 1988; 479-489

23. Leahy JL, Bonner-Weir, S. Weir GC. Rat models of non-insulin-dependent diabetes mellitus: evidence that mild increases in plasma glucose play an important role in pathogenesis. In: Lefebvre P, Pipeleers D eds. The Pathology of the Endocrine Pancreas in Diabetes 1987. Berlin: Springer-Verlag, 1988; 285-309

24. Bonner-Weir S, Leahy JL, Weir GC. Induced rat models of noninsulin dependent diabetes. In: Shafrir E, Renold AE, eds. Frontiers in Diabetes Research: Lessons from animal diabetes II. London: John Libbey 1988; 295-300

25. Leahy JL, Samols MD, Weir GC. Effects of glyburide in a rat model of reduced B-cell mass. In: Chang AY, Diani AR eds. Pathogenesis and New Approaches to the Study of Non-insulin dependent diabetes mellitus. Progress in Clinical and Biological Research Vol 265 New York: Alan R. Liss 1988; 87-98

26. Leahy JL, Weir GC. Non-insulin-dependent diabetes mellitus: Current concepts of pathogenesis. In: Draznin B, LeRoith D, Melmud S, eds. Molecular and cellular biology of diabetes and mellitus, Vol I New York: Alan R. Liss 1989; 149-158

27. Weir GC, Bonner-Weir S, Leahy JL. Pancreatic B-cell function in hyperglycemic rats: Implications for the pathogenesis of diabetes. In Ed. Cameron D. Pathogenesis of Non-Insulin Dependent Diabetes Mellitus Amsterdam, Exerpta Medica 1989; 79-84

28. Weir GC, Bonner-Weir S, Leahy JL. Effects of chronic hyperglycemia on insulin release. In: Larkins RG, Zimmet PZ, Chisholm DJ, eds. Diabetes 1988, Amsterdam Exerpta Medica 1989; 335-340

29. Gross DJ, Halban PA, Weir GC, Kahn CR, Villa-Komaroff L. Mutations of pro-insulin: effects on secetion and processing. In: Hanahan D, Cahill GF, Jr, McDevitt HO, eds. Perspectives on the Molecular Biology and Immunology of the pancreatic beta cell. Cold Spring Harbor Laboratory 1989; 43-48

30. Consensus Panel. Role of cardiovascular risk factors in prevention and treat-ment of macrovascular disease in diabetes (consensus statement). Diabetes Care 1989; 12:573-579

31. Weir GC, Bonner-Weir S. Islets of Langerhans: the puzzle of intraislet interactions and their relevance to diabetes (Perspective) J. Clin Invest. 1990; 85:983-987

32. Weir GC. Bonner-Weir S, Leahy JL. Islet mass and function in diabetes and transplantation (Perspective). Diabetes 1990; 39:401-405

33. Weir GC, Bonner-Weir S, Leahy JL. Adverse effects of hyperglycemia upon the islets of Langerhans. In Samols E. Ed. The Endocrine Pancreas. New York, Raven Press 1991:337-354

34. Weir GC, Leahy JL, Bonner-Weir S. Mechanisms which may lead to abnormal glucose induced insulin secretion in animal models of diabetes. In: Shafrir E, Ed. Frontiers in Diabetes Research. Smith Gordon and Co. London 1991; 345-349

35. Field JB, Kitabchi AE, Slovis CM, Weir GC. What to do in a glycemic crisis. Patient Care 1991; 25:81-103

36. Leahy JL, Bonner-Weir S, Weir GC. Beta cell dysfunction induced by chronic hyperglycemia: Current ideas on mechanism of impaired glucose-induced insulin secretion. Diabetes Care 1992; 15:442-455

37. Montana E, Weir GC, Bonner-Weir S. Changes in beta cell replication rate and mass in transplanted islets. Diab. Nutr. Metab. 1992; 5(suppl. 1):25-27

38. Montana E, Bonner-Weir S, Weir GC. Beta cell mass falls progressively when hyperglycemia persists after islet transplantation. Transplant. Proc. 1992; 24:2994

39. Weir GC. A defective beta-cell glucose sensor as a cause of diabetes. N. Engl. J. Med. 1993; 328:729-731 (editorial)

40. Weir GC. Current status of pancreatic and islet cell transplantation. In Current Opinion in Endocrinology and Diabetes. ed Kohler PO, Current Science, Philadelphia 1994; 226- 230

41. Weir GC. The relationship of diabetes, loss of glucose-induced insulin secretion, and GLUT2. J. Diabetes Compl. 1993; 2:124-129

42. Kinsley BT, Weir GC. Using the oral hypoglycemic agents. The Endocrinologist 1993; 3:321-9

43. Weir GC, Leahy JL. Pathogenesis of non-insulin-dependent diabetes mellitus. In: Kahn CR, Weir GC, Eds. Joslin's Diabetes Mellitus 13th edition, Lea and Febiger, Philadelphia 1994; 240-264

44. Davalli AM, Ogawa Y, Scaglia L, Wu Y-J, Hollister J, Bonner-Weir S, Weir GC. Porcine islet grafts in diabetic nude mice: Dynamic secretion and regulated B-cell mass lower glucose levels into normal porcine range. Transplant Proceedings 1994; 26:1098-1099

45. Montana E, Bonner-Weir S, Weir GC. Transplanted beta cell replication and mass increase after 95% pancreatectomy.Transplant Proceedings 1994; 26:657

46. Weir GC, Davalli AM, Hollister J, Bonner-Weir S. Islet isolation from rodents. Pancreatic Islet Transplantation Series. Volume I eds. Lanza RP, Chick WL. RG Landes Company, Austin 1994; 53-59

47. Weir GC, Nathan DM, Singer DE. American Diabetes Association Technical Review on Standards of Care for Diabetes. Diabetes Care 1994; 17:1514-1522

48. Weir GC. Glucagon-like peptide-1(GLP-1). J. Clin. Invest. 1995; 95;1 (editorial)

49. Weir GC, Singer K, Eds. Diabetes: New findings, better outcomes. Special Issue, Patient Care 1995; 29:10-120

50. Bonner-Weir SB, Davalli AM, Scaglia L, Hollister J, Weir GC. Myths about the structure and function of porcine islets. Xenotransplantation 1995; 2:207-212

51. Weir GC, Davalli AM, Ogawa Y, Scaglia L, Wu Y-J, Hollister J, Bonner-Weir S. Transplantation of porcine islets in nude mice: Implications for islet replacement therapy in humans. Xenotransplantation 1995; 2:201-206

52. Weir GC. Which comes first in non-insulin-dependent diabetes mellitus: Insulin resistance or beta cell failure? Both come first. JAMA 1995; 273:1878-1879

53. Weitgasser R, Davalli AM, Napoli R, Capotorto JV, Finegood DT, Bonner-Weir S, Weir GC. Long-term normalization of GLUT4 protein content in skeletal muscle of STZ-diabetic Lewis rats after islet transplantation. Transplantation Proceedings 1995; 27:3184-3185

54. Juang J-H, Bonner-Weir S, Vacanti JP, Weir GC. Outcome of subcutaneous islet transplantation improved by polymer device. Transplantation Proceedings 1995; 27:3215-3216

55. Davalli AM, Scaglia L, Zangen DH, Hollister J, Bonner-Weir S, Weir GC. Early changes in syngeneic islet grafts: effect of recipient's metabolic control on graft outcome. Transplantation Proceedings 1995; 27(6):3238-9

56. Weir GC, O'Hare JA. Insulin therapy and its complications. In: Becker KL, ed. Principles and Practice of Endocrinology and Metabolism. Lippencott second edition 1995; 1238-1248

57. Halban P, Weir G. Islet cell hormones: production and degradation. In: Becker KL, ed. Principles and Practice of Endocrinology and Metabolism. Lippencott second edition 1995; 1191-1197

58. Montana E, Bonner-Weir S, Weir GC. Beta cell replication and mass in islet transplantation. In. Soria B, ed. Islet Physiology and Pathophysiology. (Adv. Exp. Med. Biol.) 1997; 426:421-7

59. Bonner-Weir S, Scaglia L, Montana E, Juang J-H, Weir GC. In. Baba S, Kaneko T, eds. Diabetes 1994. Exerpta Medica, International Congress Series 1100, Elsevier. Amsterdam. Beta cell reserve: Compensatory mechanisms of the beta cell 1995; 179-183

60. Amsterdam EA, Helfand M, Schnitzer TJ, Weir GC, Young CD. Following the patient with stable chronic disease. Patient Care 1995; 29:22-42

61. Weir GC, Bonner-Weir S. Experimental Islet Transplantation. In. Lanza RP, Chick WL, eds. Yearbook of Cell and Tissue Transplantation. 1996/1997, Kluwer Academic Publishers 1996; 145-152

62. Weir GC, Bonner-Weir S. Insulin secretion in NIDDM. In. LeRoith D, Taylor SI, Olefsky JM eds. Diabetes Mellitus: A Fundamental and Clinical Text. Lippencott-Raven, Philadelphia 1996; 503-508

63. Weir GC. Back to Boston! (Editorial). Diabetes 1997; 46:1-2

64. Weir GC, Suzuki K, Trivedi N, Vasir BS, Bonner-Weir S. Engraftment issues for the transplantation of naked and macroencapsulated islets. In. Warnock GL, ed. Pancreatic islet transplantation for human diabetes: Challenges and controversies. 1997; in press

65. Weir GC, Bonner-Weir S. Islet transplantation: Why is it taking so long? Diabetes Reviews International. Diabetes Reviews International 1997; 6:2-5

66. Trivedi N, Suzuki K, Bonner-Weir S, Hollister-Lock J, Weir GC. Islet number in an immunobarrier device required to treat diabetes. Transplant. Proc. 1997; 29:2142-2143

67. Tatarkiewicz K, Hollister-Lock J, Quickel RR, Colton CK, Bonner-Weir S, Weir GC. Subcutaneous transplantation of macroencapsulated rat islets into diabetic nude mice can produce hypoglycemia. Transplant. Proc. 1998; 30:479-80

68. Yoon KH, Quickel RR, Weir GC, Bonner-Weir S. Growth and differentiation of transplanted porcine neonatal pancreatic cell clusters (NPCCs) in normal nude mice. Transplant. Proc. 1998; 30:601

69. Weir GC, Quickel RR, Yoon K-H, Tatarkiewicz, Ulrich TR, Hollister-Lock J, Bonner-Weir S. Porcine neonatal pancreatic cell clusters (NPCCs): a potential source of tissue for islet transplantation. Annals of Transplantation 1998; 2:63-68

70. Janeway CA, Ratner RE, Weir GC, Starr C. Diabetes treatment moves forward. Patient Care 1998; 32:136-147

71. Suzuki K, Colton CK, Bonner-Weir S, Hollister J, Weir GC. Estimating number and volume of islets transplanted within a planar immunobarrier diffusion chamber. Eds. Morgan JR, Yarmush ML textbook. In. Methods in Molecular Medicine, Vol 18: Tissue Engineering Methods and Protocols. Humana Press Inc, Totowa, NJ 1998; 497-505

72. Weir G, Bonner-Weir S. Islet transplantation as a treatment for diabetes. J Am Optometric Assoc. 1998; 69:727-732

73. Weir GC, Bonner-Weir S, Sharma A. Regulation of insulin secretion and islet cell function. In: Korenman SG, ed, Atlas of Clinical Endocrinology. Current Medicine, Inc. Philadelphia 2000; 1-13

74. Weir GC, Halban PA. Islet cell hormones: production and degradation. In: Becker KL, ed, Principles and Practice of Endocrinology and Metabolism. Third Edition. Lippencott, Williams and Wilkins 2001; 1296-1303

75. Weir GC. Insulin therapy and its complications. In: Becker KL, ed, Principles and Practice of Endocrinology and Metabolism. Third Edition Lippencott, Williams and Wilkins 2001 1348-1360

76. Weir GC. Pancreas and islet transplantation. In: Becker KL, ed, Principles and Practice of Endocrinology and Metabolism Third Edition. Lippencott Williams and Wilkins 2001; 1360-1365

77. Weir GC, Bonner-Weir S. Insulin secretion in Type 2 Diabetes. In. LeRoith D, Taylor SI,Olefsky JM eds. Diabetes Mellitus: A Fundamental and Clinical Text, Second Edition, Lippencott, Williams and Wilkins, Philadelphia 2000; 595-604

78. Weir GC, Bonner-Weir S. Future Trends in Diabetes Care: Transplantation. In. Wass JAH, Shalet SM, eds, Oxford Textbook of Endocrinology. Oxford University Press 2002; 1857-1866.

79. Bonner-Weir S, Weir GC. Mechanisms of postnatal β-cell mass regulation. In Habener JF, Hussain M eds. Molecular Basis of Pancreas Development and Function. Kluwer Academic Publishers 2001; 289- 297

80. Bonner-Weir S, Weir GC. Strategies for β-cell replacement in diabetes: obtaining and protecting islet tissue. In Braverman L ed. Current Opinions in Endocrinology and Diabetes. Lippencott, Williams and Wilkins, Philadelphia 2001; 8:213-218

81. Bonner-Weir S, Weir GC. How close are we to cure by islet transplantation. Diabetes Wellness News 2002; 4:1-2

82. Weir GC, Bonner-Weir S, Sharma A. Regulation of insulin secretion and islet cell function. Second Edition. In: Skyler JS, ed, Atlas of Clinical Endocrinology. Current Medicine, Inc. Philadelphia 2002; 1-13

83. Cefalu WT, Weir GC. New technologies in diabetes care. Patient Care September 2003; 66-78

84. Weir GC, Haagensen A, Bonner-Weir S. Insulin-producing cells derived from embryonic stem cells: A potential treatment for diabetes. In R Lanza, ed. Handbook of Stem Cells, Volume 1: Embryonic Stem Cells. Elsevier 2004; 723-730

85. Weir GC, Bonner-Weir S: Insulin secretion in type 2 diabetes. In Diabetes Mellitus, A Fundamental and Clinical Text,. 3rd Edition. Edited by LeRoith D, Taylor SI, Olefsky JM. Philadelphia: Lippincott Williams & Wilkins. 2004: 887-898

86. Bonner-Weir S, Weir GC. Adult progenitor cells as a potential treatment for diabetes. In R Lanza, ed. Handbook of Stem Cells, Volume 2: Adult and Fetal Stem Cells. Elsevier 2004; 731-738

87. Weir GC, Bonner-Weir S, Sharma A. Regulation of insulin secretion and islet cell function. In: Skyler JS, ed, Atlas of Clinical Endocrinology. Third Edition. Current Medicine, Inc. Philadelphia 2004; 1-13

88. Weir GC, Bonner-Weir S. Insulin-Producing Cells Derived from Stem Cells: A Potential Treatment for Diabetes. Essentials of Stem Cell Biology (Robert Lanza, Coordinating Editor) Elsevier 2005; 411-418

89. Weir GC, Gaglia JL. Pancreatic and Islet Transplantation. In DeGroot L and Jameson L Endocrinology Fifth Edition, Elsevier Saunders 2005; 1271-1292

90. Colton CK, Papas KK, Pisania A, Rappel MJ, Powers DE, O'Neil JJ, Omer A, Weir G, Bonner-Weir S. Characterization of Islet Preparations. Cellular Transplantation: From Laboratory to Clinic. Elsevier, 2007; :85-133

Monographs

1. Bonner-Weir S, Weir GC. The islets of Langerhans and diabetes mellitus. Current Concepts Series, Upjohn 1986 (authors)

2. Kahn CR, Weir GC, Eds. Joslin's Diabetes Mellitus 13th edition, Lea and Febiger, Philadelphia 1994

3. Kahn CR, Weir GC, King GL, Jacobson AM, Moses AC, Smith RJ, Eds. Joslin's Diabetes Mellitus 14th edition, Lippincott, Williams and Wilkins 2005